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Practical Approach to Mammalian Cell and Organ Culture

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Practical Approach to Mammalian Cell and Organ Culture

With 261 Figures and 89 Tables

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ISBN 978-981-19-1730-1 ISBN 978-981-19-1731-8 (eBook)
https://doi.org/10.1007/978-981-19-1731-8

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

The mammalian body is comprised of many diverse systems. These systems, in turn, are composed of different organs, which are further made up of various tissues, each of which is made up of numerous cell types, all coming together to form the structural and functional units of life. Cells represent fundamental components of tissues and organs from both a structural and functional perspective. Isolation of cells from their respective tissues as well as their subsequent in vitro culturing is the first step in understanding in vivo structural and functional organ framework.

Culturing mammalian cells, tissues, and organs comprises a key domain of modern biomedical research. In particular, mammalian cell culture is necessary for the production of recombinant proteins, enzymes, vaccines, antibodies, and hormones. This book, entitled *Handbook of Practical Approach to Mammalian Cell and Organ Culture*, describes the various aspects of a mammalian cell, tissue, and organ culture both on a small scale for a research laboratory as well as on an industrial scale.

Substantial literature is already available on mammalian cell culture. Unfortunately, most of this information lacks simplicity and in-depth analysis of practical research protocols needed for early-phase investigators although most of the sources elaborate on the general principles of cell culture and lack the details for a particular cell culture protocol. In mammals, any tissue typically is comprised of numerous cell types and associated extracellular matrix (ECM) with organ-specific varying compositions. Thus, cell isolation and culture protocols vary from organ to organ. Moreover, specific experiments may require pre-treatment with drugs, toxins, growth factors or cytokines, etc. These details are frequently missing in the current literature. Additionally, there is a lack of an in-depth presentation of all the aspects of mammalian cell culture and its applications.

In the last century, significant progress has been achieved in mammalian cell culture, including the isolation and culture of single mammalian cells. Biological science has progressed from culturing mammalian cells and tissues from two-dimensional (2D) to three-dimensional (3D) environment. Parallel developments have emerged in culturing mammalian organs, organoids, and organs-on-a-chip. A comprehensive discussion commencing from 2D, 3D, and finally to organ culture and infusion of mammalian cell culture technology to biomedical engineering, particularly tissue engineering, biosensors, and gaining better control using

nanomaterials and IPR prospect of mammalian cell culture, is therefore needed. These needs are addressed in this book.

The central goal of this book is to provide a comprehensive approach via isolation protocols, including single-cell isolation and specific treatment conditions for the most widely studied primary cells including cells obtained from humans (*Homo sapiens*) and mice (*Mus musculus*). In addition, in separate chapters, culture and propagation of various secondary cultures, i.e., established mortal and immortal cell lines using 2D and 3D formats, are also discussed. Description of common cell-based assays, the large-scale culture of mammalian cells using instruments such as bioreactors for industrial purposes, and the usefulness of mammalian cells in various aspects of the biomedical industry including vaccines, antibodies, and recombinant protein production are other prominent features of this book. Finally, the book is completed by adding some diversified chapters such as the reliability of mammalian cells as a model system in the ecotoxicological evaluation of environmental stressors and the usefulness of bioimaging in modern research. Thus, the book provides a comprehensive review of current mammalian cells, tissues, and organ cultures techniques and their various applications.

Each chapter contains an updated introduction and a reference list designed specifically to provide the readers with robust content summaries and sources. Finally, a comprehensive review of mammalian cell culture systems provides access to detailed protocols, cell culture assay, and troubleshooting that will hopefully assist a broad spectrum of readers and investigators including cell biologists, immunologists, virologists, biotechnologists, pharmacologists, cancer biologists, stem cell researchers, medical practitioners, and animal biotechnologists. We hope that through our collective efforts, this book will be useful to both students and teachers.

Noida, India — Tapan Kumar Mukherjee
Gandhinagar, India — Parth Malik
Chandigarh, India — Srirupa Mukherjee
March 2023

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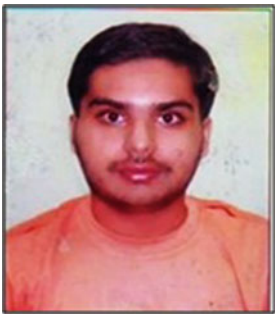
About the Editors

Dr. Tapan Kumar Mukherjee completed his graduation and post-graduation in Human Physiology with 1st class from Kolkata University, India. He has a Ph.D. in Human Physiology from Bose Institute, a Department of Science and Technology (DST), Government of India organization. Following his Ph.D., Dr. Mukherjee further enriched his research expertise during his post-doctoral stints at the Center for Cellular and Molecular Biology (CCMB), Hyderabad, India, and the Department of Molecular and Medical Pharmacology, University of California, Los Angeles (UCLA). He has faculty experience from internationally renowned Institutes like the University of Utah, Salt Lake City, USA, Indian Institute of Science Education and Research (IISER), Mohali, Punjab, India, and Maharishi Markandeshwar University, Mullana, Haryana, India. Presently, he is working as a Professor at the Institute of Biotechnology, Amity University, Noida, UP, India. Dr. Mukherjee has visited the Korea Research Institute of Bioscience and Biotechnology (KRIBB), South Korea, and the National University of Singapore (NUS), Singapore. He has been awarded a national scholarship from Kolkata University and DST post-doctoral fellowship (sponsored) at CCMB, Hyderabad, India. On the research front, his primary interest is deciphering the cell signaling molecules involved in the complication of cancer and cardiovascular diseases. His expertise involves the application of Molecular Medicine Protocols to control these diseases. Dr. Mukherjee has received several research grants from the Indian Council of Medical Research (ICMR), the Department of Biotechnology (DBT), and the Department of Science and Technology (DST), the

Government of India. He has several research publications in first-rated international journals including three in *PNAS* (USA), *Journal of Biological Chemistry*, *Journal of Immunology*, *Cellular Oncology*, *Nanobiotechnology*, and *Life Sciences*, and several in *Biochemica et Biophysica Acta* (BBA) Molecular Cell Research, *Biochemical Journal*, *Infection Immunity*, *BBRC*, etc. He has also contributed to writing Encyclopedias from Springer and Elsevier and book chapters in international publication houses like Springer, Nova Science Publishers, Pan Stanford Publishers, and Bentham Science. He has trained numerous students and Ph.D. candidates. His dedication, devotion, and undisputable academic consistency earned him the best teacher award.



Dr. Parth Malik holds a graduation in Biotechnology and a post-graduation in Nanobiotechnology interface (domain). After being awarded M.Tech. in 2011, he devoted a year to sharpening his teaching and research skills in the Department of Biotechnology, Maharishi Markandeshwar University, Mullana (Ambala, India). It was in this duration that Dr. Malik enhanced his scientific writing and creative thinking in the domain of "Understanding biochemical significance and functional correlations of free radicals and antioxidants," aroused by manifold encouraging discussions with Prof. Mukherjee. Subsequently, he left for Central University of Gujarat in 2012, where he completed his M.Phil. and Ph.D. in the School of Nano Sciences, in 2018. He has a rich experience in the preparation of low energy-based oil-in-water emulsions to enhance the antioxidant expressions of natural bioactive compounds. His major research work is on optimizing the antioxidant efficacy of polyphenolic antioxidant curcumin, via nano- and microemulsions of cottonseed, peanut, mustard, and linseed oils. His association with Prof. Mukherjee as well as with Prof. Man Singh (Ph.D. supervisor) is the reason for his cemented expertise in the subject areas of antioxidants and free radical dynamics and Physicochemical characterizations of emulsions, respectively. Dr. Malik has attained success in publishing his research works on curcumin nanoemulsions with variable surfactants, characterized by their temperature-optimized

physicochemical activities, and developing the most authentic structure-activity relationships. Two primitive aspects of his research work comprise developing solutions to low bioavailability-hindered impaired structural expression of natural polyphenols through facilitating their sustained expression and exploring the commonality of physicochemical modulations with structural expressions of dispersed phases. It is no more a surprise now that efficient (> 70%) free radical scavenging activities could be attained even with >1000 nm particle sizes, where self-assembly-driven molecular controls could supersede. His overall research expertise comprises overlapping boundaries of oxidative stress mitigation, nanoemulsions preparation and optimization for improving the structural expression of low bioavailability compounds, physiochemical interpretations of dispersion stability, nanobiotechnology, biosensors, and the use of biocompatible and structurally robust materials to improve dispersion potential of emulsions.

Dr. Srirupa Mukherjee completed her post-graduation in Biochemistry with 1st class from Kolkata University, India. Subsequently, she was awarded a Ph.D. from the Indian Institute of Chemical Biology (IICB), a Council of Scientific and Industrial Research (CSIR) Organization, Government of India. She has availed of Junior and Senior Research Fellowship (JRF and SRF) grants from CSIR, Government of India. Dr. Mukherjee has several years of successive post-doctoral training experience, from the University of Iowa, the University of California, Los Angeles (UCLA), and the University of Utah, USA. After completing her post-doctoral training, Dr. Mukherjee returned to India and joined the Indian Institute of Science Education and Research (IISER), Mohali, as a Senior Research Associate. Presently she is working as a DST Women Scientist at the Post-Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Dr. Mukherjee has expertise in several research domains comprising host-pathogen interactions, atherosclerosis, and respiratory biology. She has also written several book chapters in eminent books reviewed and edited by renowned global experts associated with reputed international publishers. She has several international publications in eminent scientific journals, encompassing

Vaccines, *Atherosclerosis Thrombosis and Vascular Biology* (ATVB), *Biochimica et Biophysica Acta* (BBA), *Journal of Parasitology*, *Journal of Immunology*, *Respiratory Research*, *Clinical and Vaccine Immunology* (CVI), etc. She has trained numerous project fellows, and several Ph.D. students are perusing their research work under her able guidance.

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Mammalian Cell Culture: An Overview

Srirupa Mukherjee, Parth Malik, and Tapan Kumar Mukherjee

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_1-1

Abstract

The mammalian body typically consists of trillions of cells. These cells are the basic structural and functional units of a mammalian body. Together, these cells form tissues, tissues form organs, and organs collectively build up systems, constructing a whole body. The basis of cell theory states that mammalian cells originate from the pre-existent mother cells by the simple process of cell division (mitosis/meiosis). Thus, cell culture not only aims toward understanding the basic steps necessary for physiological processes involved in regular homeostasis in the body but also understanding various pathophysiological or diseased conditions associated with cellular dysfunction and their regulation by synthetic drugs/toxins, etc. Technically, **mammalian cell culture** is defined as the ongoing process by which cells are isolated from a mammalian body (typically from a specific tissue or organ) and are grown in vitro with a constant supply of synthetic nutrients under controlled laboratory conditions of optimum pH, temperature, and humidity in a CO_2 incubator/bioreactor. Nowadays, **the term tissue culture is often used interchangeably with cell culture**. In modern times, cultured mammalian cells are widely used for the bulk production of antibodies, vaccines, cytokines, growth factors, hormones, and other proteins in demand. To understand mammalian cell culture, it is not only important to gain knowledge regarding the basic concept and the ardent contributions of various scientists towards the development of this unique technology but it also requires selecting and validating specific experimental conditions for cell culture.

Keywords

History of mammalian cell culture · Karyotyping and authenticity of mammalian cells · Selection and validation of mammalian cells · Cell cycle and cell division in eukaryotes · In vitro culture of mammalian cells · Cell synchronization

1 Introduction

Evolutionary mammals are the most developed living creatures on this planet earth. Humans or *Homo sapiens* are positioned at the top of this evolutionary hierarchy. A mammalian body consists of trillions of cells that form the structural and functional foundations. (A typical human body may contain 30–40 trillion cells). Various cells form the tissues; tissues combine to generate an organ, and organs form an organ

system, which ultimately gathers and arranges together to give rise to a complex mammalian body (Martini and Nath 2009; Alberts et al. 2013).

It has been the eternal curiosity of the human mind to understand the genesis of new cells in a mammalian body. It is the handy proposal of ***Rudolf Virchow***, who modified cell theory, previously postulated by ***Matthias Jacob Schleiden*** and ***Theodor Schwann***, with Virchow's hypothesis being, "all organisms are composed of one or more cells and all cells come from preexisting cells." This fundamental concept indicates that **cell division** is the only process through which any living creature (including mammals) can get new cells (Silver 1987; Mazzarello 1999; Bradshaw and Stahl 2015).

In a cell, DNA synthesizes DNA by the process of **replication**, DNA synthesizes RNA by the process of **transcription**, and RNA synthesizes protein by the process of **translation**. This replication, followed by transcription and subsequently translation, is known as the **Central Dogma of Molecular Biology**. For a mammalian cell to divide (proliferate), every component, including DNA, RNA, and proteins, must be exactly doubled by the patterned events of replication, transcription, and translation, respectively. All the somatic cells (those which are not involved in reproduction) within a particular body are genetically exactly similar. However, within a cell at a particular time, certain genes may remain **transcriptionally switched on (or active)**, contrary to others that remain inactivated. This switching on or switching off of genes (**transcriptional regulation**) in somatic cells differs from one to another cell type. Thus, **due to differential expression** patterns, **mRNAs** and **proteins** expressed by one somatic cell may be absent in another somatic cell. Changes in the protein expression extents are known to be responsible for s**tructural (phenotypic)** and **functional** variations from one to the other somatic cell type (Crick 1970; Weber et al. 2007).

In vitro culturing of cells, tissues, and organs are one of the technologies that aid in a better understanding of the physiology and pathophysiology of the mammalian body. **Mammalian cell culture** is defined as the process by which cells are isolated from a mammalian body (specific organ or tissue) and are grown in vitro under controlled laboratory conditions of maintained **pH, temperature, humidity, O_2/CO_2** tension, and **readymade nutrient supply** (through cell culture medium). **Tissue culture** is the collective process by which whole tissues are derived (dissected) from a mammalian organ and are grown in vitro under controlled environmental conditions, either on a semi-solid, solid (**agar**), or liquid (**nutrient broth**) culture medium, supplemented with nutrients and growth factors, similar to that of cell culture. **Nowadays, the terminologies of tissue culture and cell culture are often used interchangeably** (McLimans 1972; Jacoby and Pasten 1979; Harris 1993; Clynes 1998; Mather and Barnes 1998; Davis 2011; Wilmer 2013).

This chapter describes the fundamentals of the discovery of cell/tissue culture techniques in multicellular eukaryotes, i.e., mammals. The year-wise chronological summary of major cell and tissue culture discoveries is cited in this chapter. Towards the end, the chapter sheds light on the factors necessary for the selection and validation of desired cells for one or more specific purposes.

2 Concept of Cells, Tissues, and Organs

Mammals are multi-cellular animals having eukaryotic (**EU** means developed, **KARYON** means nucleus) cells. Anatomically and physiologically, a mammalian body comprises different systems that are subdivided into various organs. Organs are further subdivided into more than one type of tissue. A particular type of tissue is composed of multiple similar types of cells. A cell is recognized as a fundamental structural and functional unit of life.

Ahead is a brief description of mammalian body constituents.

2.1 Cells of a Mammalian Body

Cells are the structural and functional units of all living organisms, including mammals. The term cell was first introduced by ***Robert Hooke*** in **1665** while observing the section of a dead cork through a simple microscope discovered by ***Anton van Leeuwenhoek***. In comparison to prokaryotic cells (**PRO** means primitive, **KARYON** means nucleus), mammalian cells are very **large**, with an average size ranging from (10 to 100) μm in diameter. Red blood cells (RBCs), one of the smallest human cells, have a diameter of around 8 μm and mature human RBCs do not have a nucleus. In the human body, the **longest cell** is the nerve cell. The **largest cell** in the **human** body is the female ovum. Male sperm is a very small cell. Regarding **shape**, mammalian cells exhibit significant diversity. In general, the cells are elongated or spherical. Some cells are long and pointed on both ends. Such cells exhibit a spindle **shape** (e.g., smooth muscle cells). In some cases, the cells could be very long. Some may be **branched** like the neurons or the nerve cells. In humans, there are about **200 different** cells and within these cells, there are about **20 different** structures or organelles (Schierbeek 1959; Hooke 1665; Gest 2004).

2.2 Tissues of a Mammalian Body

The word **tissue** means a group of cells that have a similar structure and act together to perform a specific function. The word **tissue** originated from a form of an old French verb meaning ***to weave***. A particular type of tissue contains a similar kind of cells. For example, in epithelial tissue, only various cells are present. In all multi-cellular organisms, cells, matrices, fibers, and many other materials help to form a tissue (Bryant and Mostov 2008).

The following four types of tissues form various mammalian organs:

Epithelial tissue
Connective tissue
Muscular tissue
Nervous tissue

Here is a brief presentation of the various tissues:

2.2.1 Epithelial Tissue

Marie François Xavier Bichat discovered epithelial tissue between 1771 and 1802. He was a French anatomist and pathologist and was subsequently remembered as the ***Father of Histology***. The epithelial tissue is made up of epithelial cells. Based on the specific shape, epithelial cells could be divided into **squamous, columnar**, and **cuboidal**. **Epithelial cells** are present in the outer layer of various body structures, including skin, organs, cavities, blood vessels, etc. The cells in epithelial tissue are tightly packed together with a very little intercellular matrix. Epithelial layers are **avascular** but **innervated**. The primary function of the epithelial tissue is the **protection** of all structures on the outer surface on which they reside. Other major functions of epithelial tissues are **secretion**, **absorption**, **excretion, filtration, diffusion,** and **sensory reception (**Fig. 1 describes various kinds of epithelial cells present in the human body).

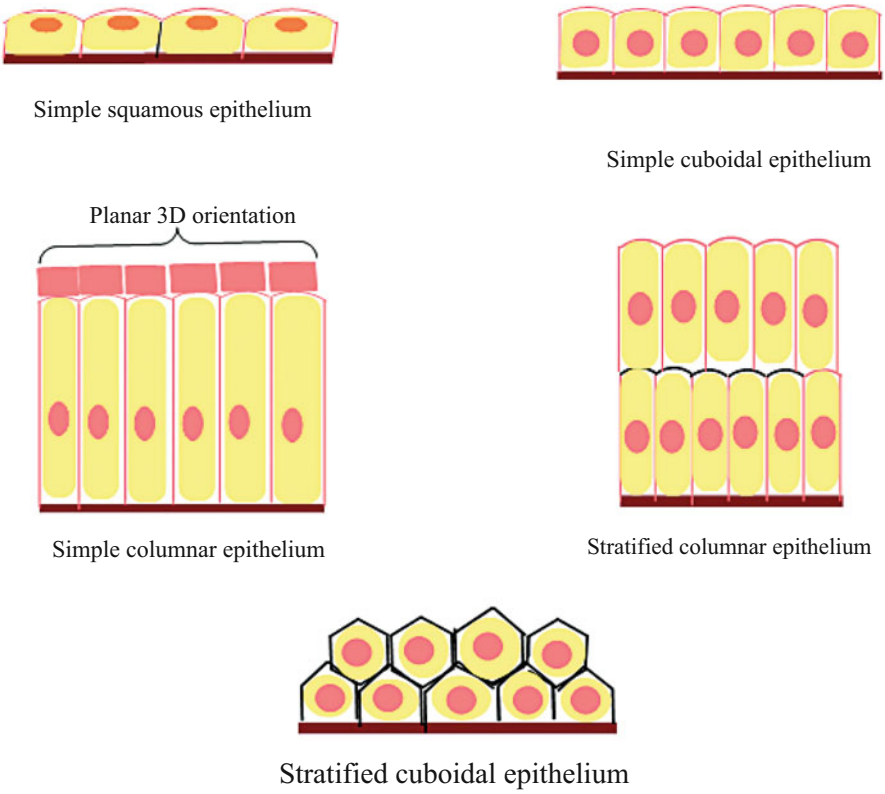


Fig. 1 Types of epithelial tissues lining body systems

2.2.2 Connective Tissue

Connective tissue is one of the four basic types of mammalian **tissue**. It develops from the **mesoderm of the embryo**. All connective tissue consists of three main components: **fibers, ground substance,** and **cells.** There are three types of fibers in the intercellular matrix of connective tissue: **collagen fibers, reticular fibers, and elastic fibers**. The most prevalent protein in the body is **collagen**, constituted of a triple helix structure. In ordinary connective tissue, the ground substance consists of water stabilized by glycosaminoglycans, proteoglycans, and glycoproteins. In bones, the ground substance includes minerals. In blood, the ground substance is **fluid** (plasma). The major cells present in connective tissue are fibroblasts, adipocytes, and various blood cells (e.g., RBC, WBCs, and platelets). **Blood is often called the liquid connective tissue**. As the name suggests, the basic function of connective tissue is to connect and communicate within cells, tissues, organs, and systems of a mammalian body. Other functions of connective tissues are binding and supporting, protecting, insulating, storing reserve fuel, and transporting substances within the body (Fig. 2 represents the fibrous connective tissue and blood, the liquid connective tissue).

2.2.3 Muscular Tissue

A type of tissue found in the mammalian body which functions by contracting, thereby exerting forces on different body parts. Muscle tissue consists of fibers of **muscle cells** connected in sheets and fibers. The muscle tissue is made up of **cardiac muscle** (present in the heart, semi-striated), **skeletal muscle** (attached to the skeleton, striated), and **smooth muscle** (present in the wall of the hollow visceral organ, non-striated). While cardiac and smooth muscles are involved in involuntary contraction, a skeletal muscle is involved in voluntary contraction. At the cellular level, each muscle cell has a complex of proteins containing **actin** and **myosin**. These proteins slide past one another when the contraction signal is received (**known as sliding filament theory, conceived by Hugh Huxley in 1953**). The filaments are connected to the ends of cells, and as they slide past one another, the cell contracts in length. A single cell can contract up to 70% in length, shortening the entire muscle in the process. Muscle tissue can be used to move bones, compress chambers, or

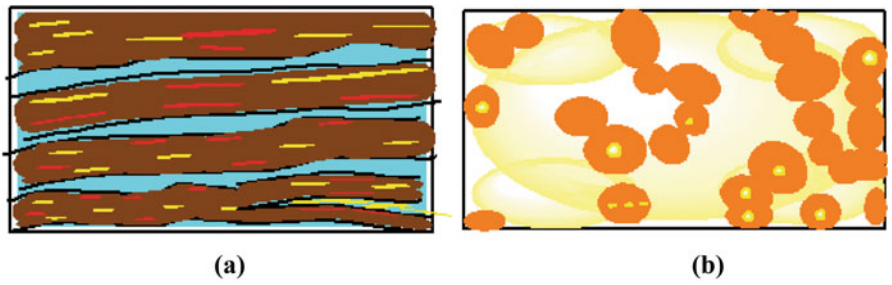


Fig. 2 Schematic representations of (**a**) fibrous connective tissue and (**b**) blood, the liquid connective tissue

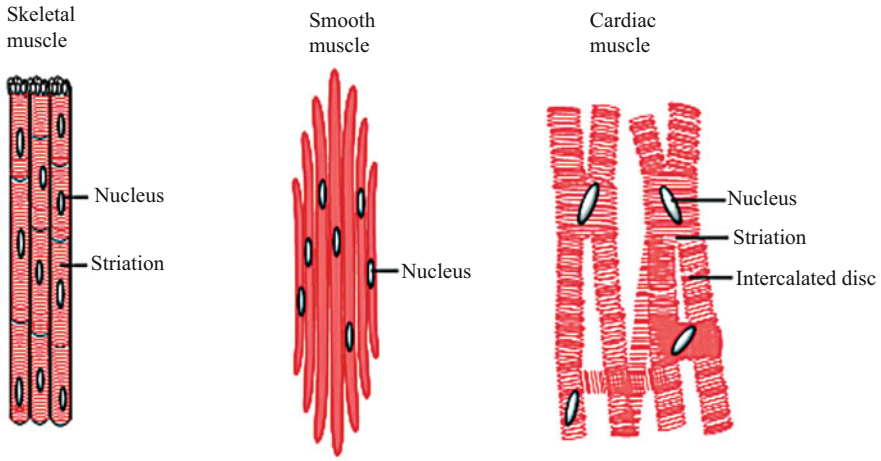


Fig. 3 Schematic description of muscular tissue diversities

squeeze various organs (Fig. 3. represents the three kinds of muscular tissues, i.e., skeletal muscle, smooth muscle, and cardiac muscle).

2.2.4 Nervous Tissue

Nervous tissue or neuronal tissue is a tissue that is made up of **neurons** or **nerve cells** and **neuroglial cells** (also called **supporting cells**). These cells are present in the central and peripheral nervous systems. A typical neuron consists of **Dendron and dendrites**, **the cell body (also called soma)**, and an **axon**. Dendrons and dendrites are responsible for responding to stimuli and receiving incoming signals towards the cell body. Dendrons and dendrites are motor neurons that are short and have a large surface area for receiving signals from other neurons. These convey incoming messages toward the cell body and are therefore called the receptive input regions. The cell body is like a factory for the neuron, producing all the proteins and containing specialized organelles such as the **nucleus, granules, and Nissl bodies.** The axon arises from the cone-shaped portion of the cell body called as **axon hillock**. Functionally, the axon is the conducting region of a neuron and is responsible for generating and transmitting impulses, typically away from the cell body. A single axon routes the nerve impulse from the cell body to another neuron or an effector organ. The axon can have many terminal branches, whereby there are chances of more than one cell getting stimulated each time, and nerve fibers. The axons are responsible for transmitting impulses over long distances from the cell body. The axon may be either **medullated** (covered with myelin sheath) or non-medullated (without myelin sheath). In the modulated axon, some regions of the myelin sheath are broken, which are known as **Nodes of Ranvier**, whereas in medullated nerve, ions only pass through the Nodes of Ranvier.

There are **six types of neuroglial cells**: four in the central nervous system **(CNS)** and two in the peripheral nervous system **(PNS)**. These glial cells are involved in

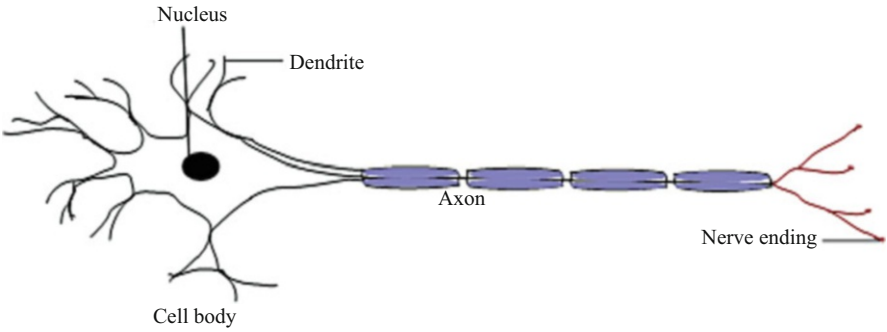


Fig. 4 Typical structure of a neuron, the functional unit of the nervous tissue

many specialized functions apart from the support of the neurons. Neuroglia in the CNS includes **astrocytes, microglial cells, ependymal cells, and oligodendrocytes**. In the PNS, **satellite cells and Schwann cells** are the two kinds of neuroglia. While neurons are highly specialized nerve cells that generate and conduct nerve impulses, neuroglias are supporting cells that provide physical support, remove debris, and provide electrical insulation. Thus, integration and communication are the two major functions of nervous tissue (Fig. 4 depicts a complete neuron).

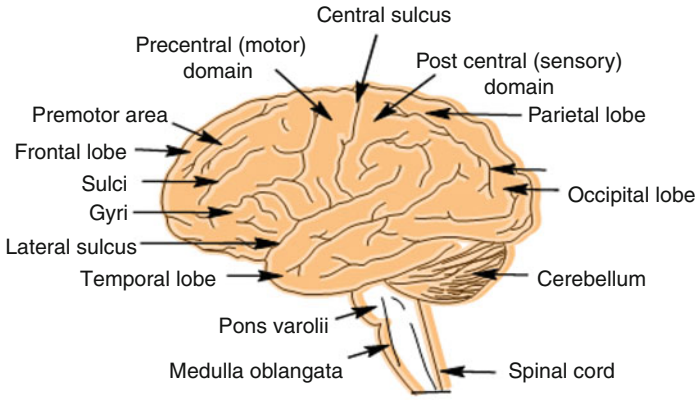
2.3 Organs of a Mammalian Body

Various tissues link together to form an **organ**.

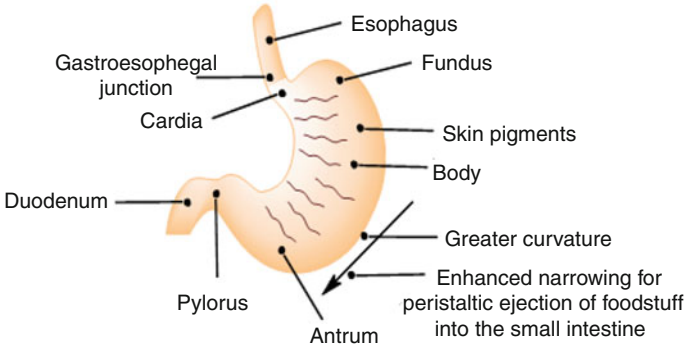
The major vital organs of a mammalian body and their functions are as follows:

- The **Brain** controls thoughts, memory, and other organs.
- The **Heart** pumps blood throughout the body.
- The **Lungs** separate oxygen from the air and remove carbon dioxide from the blood.
- The **Stomach** helps to digest food.
- The **Intestines**, that aid in nutrient absorption from food.
- The **Liver** removes/breakdowns poisons (metabolites) from the blood.
- The **Kidneys**, filter blood and produce urine.
- The **Bladder** stores urine.
- The **Skin** guards the body and contains other organs.

The generalized structures of various vital human organs are depicted through different subsections of Fig. 5**.**



(a)



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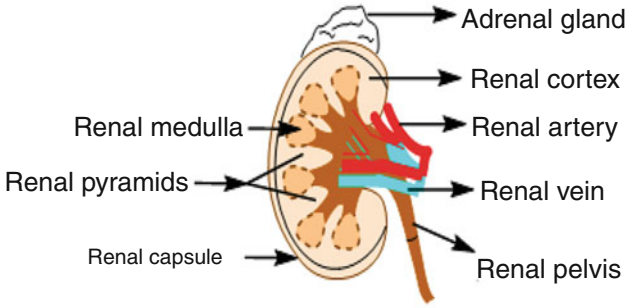
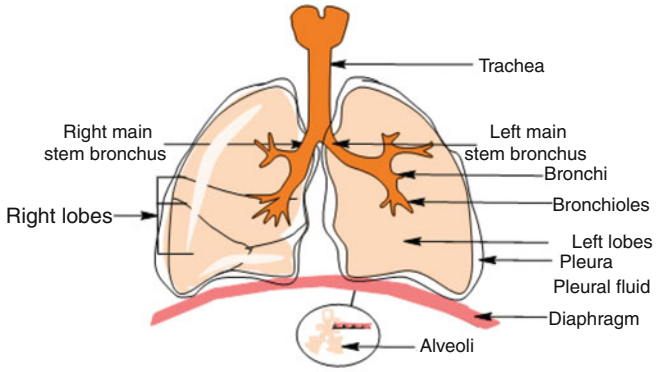


(c)

Fig. 5 Generalized structures of vital human organs, in a chronological manner as, (**a**) Brain, (**b**) Stomach, (**c**) Kidney (cross-sectional view), (**d**) Lungs (respiratory system), (**e**) intestines (digestive system), (**f**) liver, (**g**) gall bladder and (**h**) heart



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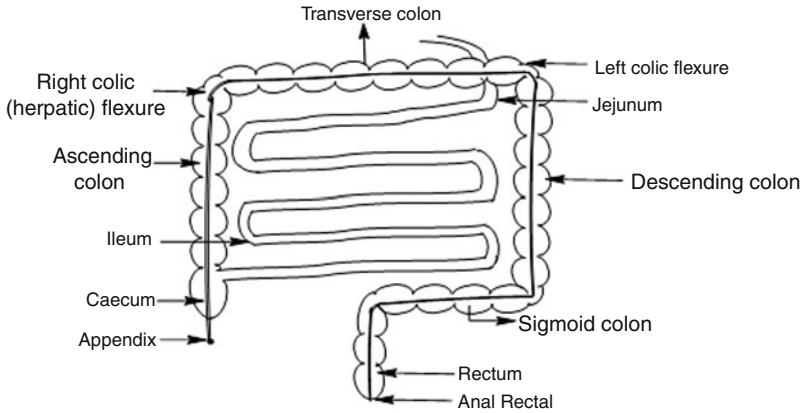

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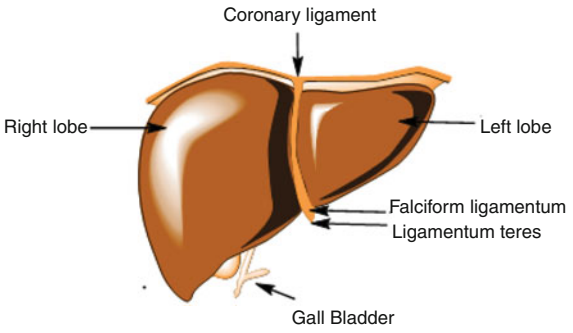

(f)

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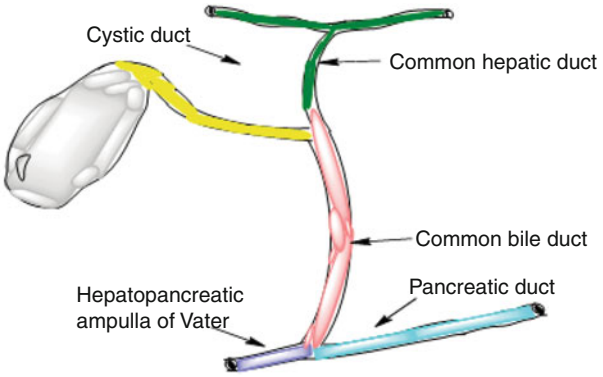

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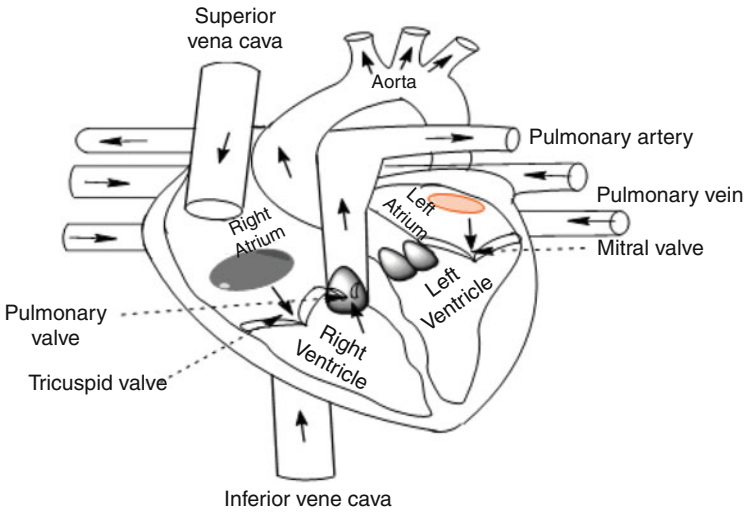

(h)

Fig. 5 (continued)

2.4 Organ Systems of a Mammalian Body

In a mammalian body, various organs are linked together to form a **system** (e.g., cardiovascular system consists of the heart, arteries, veins, and capillaries).

The major organ systems of the human body are as follows:

- **Nervous System**

It consists of cell body (Soma) and afferent and efferent neurons. It receives nerve signals, analyzes them, and sends a specific response to the effector organs.

- **Circulatory System**

It includes the heart, veins, arteries, and capillaries. Its function is to transport substances (e.g., nutrients, O_2, CO_2, metabolites, etc.) in the blood, throughout the body.

- **Respiratory System**

It includes the nose and the lungs. It takes in oxygen and removes carbon dioxide.

- **Digestive System**

It includes the mouth, esophagus, stomach, and small and large intestines. It breaks down food to absorb nutrients and water. It is also extended to the colon through which undigested food materials are passed out as stool.

- **Reproductive System**

It includes the vagina, uterus, fallopian tube, and ovary in women, and the penis, vas deference, and testes in men. Its function is to generate offspring via facilitating male and female gametes during reproduction.

- **Musculoskeletal System**

It includes bones and muscles. It supports the body and allows movement.

- **Immune System**

It includes bone marrow, thymus, spleen, lymph nodes, MALT, GALT, and immunological cells. It protects the body from infection and is described as a defense system of the human body.

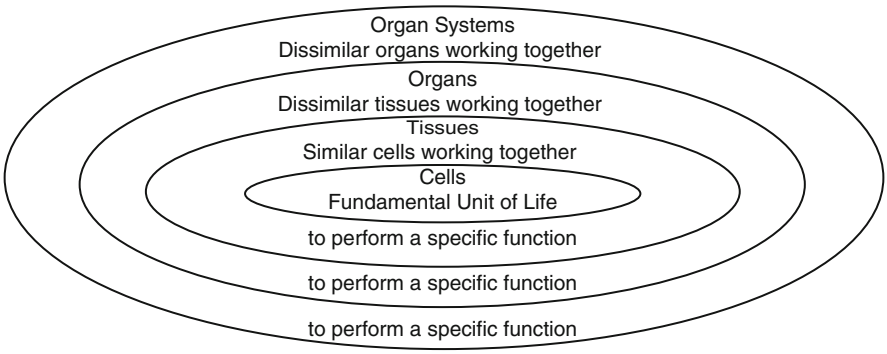

Fig. 6 Hierarchical make-up of the mammalian body, comprising of cells, tissues, organs, and organ systems. The organ systems collectively form a complete body, which works as a single unit

2.5 A Complete Mammalian Body

The physical substance of all the organ systems constitutes a complete mammalian body. Thus, a whole **body** is nothing but a superb arrangement of the various organ systems. Therefore, the formation of a whole-body system from cells, tissues, and organs, and finally organ systems, is the central theme of both ***mammalian anatomy and physiology.***

The process through which cells form a mammalian body is depicted in Fig. 6***, as follows:***

3 Basics of Cell Theory

Most cells are very small in dimension and are, therefore, visible only under a microscope. It is claimed that sometime around 1590, two Dutch spectacle makers, ***Zacharias Janssen*** and his father ***Hans***, started experimenting with lenses. They put several lenses in a tube and could able to see small molecules that cannot be seen with the naked eye. However, it was ***Antonie Philips van Leeuwenhoek*** whose microscopic observation of cells remains a breakthrough accomplishment in **Cell Biology**. A **microscope is considered** one **of the most essential instruments in a cell culture laboratory. Using this microscope, *Robert Hooke* first observed the cells**. Thus, the discovery of the microscope led to the discovery of cells, and sub-cellular structures, laying the foundation for understanding their fundamental behavior and characteristics. Cell theory comprises the gathering of all the meticulous research works in this field (Schierbeek 1959; Hooke 1665; Gest 2004; Masters 2008).

The theory was first developed in **1839** by ***Matthias Jakob Schleiden*** and ***Theodor Schwann*** and was later modified by ***Rudolf Virchow*** in **1858** (Alberts et al. 2013).

At present, the basic essence of cell theory revolves around the following important points:

- All organisms are composed of one or more cells, and **all cells come (originate) from pre-existing cells**.
- In an organism, all basic functions occur inside the cells, and all cells contain hereditary information for performing these basic functions.
- The hereditary information from chromosomes (DNA) and genes are transmitted to the next generation by the process of mother cell division into new daughter cells.

While cell theory described some important fundamental cell characteristics, it did not mention that the mammalian body comprising of not just one but two types of cells, **i.e.,** ***somatic and germ cells, and their division procedures may slightly differ.***

4 The Somatic and Germ Cells

A typical human body may have 200 different kinds of cells. Functionally, mammalian cells are divided into **somatic** and **germ (reproductive)** cells. Somatic cells form the whole body of a mammal except the reproductive organs. It indicates that every organ of a mammal is made up of somatic cells except those of the testes in males and ovary in females, which produce the reproductive or gamete or germ cells. The germ cells of testes are responsible for the production of **sperm in males**, while the germ cells in female (**Graafian follicle**) produce ovum. The somatic cells of humans contain a double number of chromosomes **(diploid, for humans it is 46)** compared to that of sperm or ovum, which contain half the number of chromosomes **(haploid, for humans it is 23)** to that of somatic cells. Different mammals (rat, mouse, human, etc.) may have dissimilar chromosome numbers in their cells. However, all somatic cells within the body of a particular species possess an exactly equal number and type of chromosomes. It indicates that every single human throughout the world contains exactly 46 chromosomes in each of their somatic cells and 23 lies in each reproductive cell. Of note, according to the **human genome project**, a typical human somatic cell may contain around 20,000 to 25,000 genes and may have an identical number of proteins. However, in the mammalian body, since genes may be present in several alternative forms, the actual number of proteins from a typical mammalian cell may be as high as 100,000. Interestingly, various genes may be functional (producing proteins) in different cells at the same instant.

Fundamentally, different phenotypes (morphology) and functional characteristics of various mammalian cells depend on the nature of **specific proteins expressed** by a particular mammalian cell. This further indicates that due to the **differential expression of various genes,** one type of somatic cell (e.g., neuron) may be morphologically, structurally, and functionally distinct from that of the other (e.g., smooth muscle cells). For example, while a neuronal cell looks like a tree and transmits a neuronal signal, a smooth muscle cell has a spindle shape and is involved in involuntary contraction. Every phenotypic characteristic is a reflection of the specific proteins produced by a particular cell type.

The fundamental rules of cellular characteristics are violated due to **spontaneous or induced genetic alterations (mutations) or epigenetic (beyond genetic) alterations, such as alteration in the pattern of DNA acetylation or methylation**, leading to the morphological and functional alterations of cells. This fundamental alteration gives rise to new types of cells, i.e., tumor or cancer cells. Of note, mutations in the germ cells are only inherited by the next generation, while somatic cell mutations only affect their implicit host and not the next generation.

Therefore, cells can be characterized based on their **karyotype (chromosomal characteristics)** and **morphology** (Puck and Fisher 1956; Nicholls et al. 2019).

5 Characterization of Cells: Karyotype and Morphological Analysis

Normally, mammalian cells are characterized by karyotype analysis and morphology.

5.1 Karyotype Analysis

The chromosomal characteristics of mammalian cells can be determined by karyotyping, a technique discovered by ***Levitsky***. Karyotyping determines the origin of a species and ascertains the extent of gross chromosomal changes in a cell line. Karyotypes describe the chromosome count of an organism and what these chromosomes look like under a light microscope. One must focus on their length, position of the centromere banding pattern, the differences between the sex chromosomes, and any other physical features. The preparation and study of karyotypes come under the umbrella of cytogenetics. The study of whole sets of chromosomes is sometimes known as **karyology**. The karyotype of cells is affected by the growth conditions, the pattern of cellular growth, and whether or not, the cells are preserved in the frozen state. Karyotypes can be used for many purposes, such as to study chromosomal aberrations, cellular functions, taxonomic relationships, and medicine and to gather information about past evolutionary events (Levitsky 1931; MacLeod and Drexler 2005).

5.2 Morphology Analysis

Most mammalian cells are morphologically sub-divided into three basic categories.

5.3 Epithelial

These cells are derived from all three germ layers, though the true epithelium is considered the **ectoderm of the growing embryo**. These cells are **polygonal and flattened** with more regular dimensions and grow as being attached to a substrate in discrete patches. Mostly, these cells could exist in **squamous** (flat or scale-like), **cuboidal** (height and width are the same), or **columnar** (cells are taller than wide) morphology.

Epithelial cells develop into sheets or tubes that separate an organism from its environment, allowing for the survival of multicellular organisms. Tissues encoding epithelial cells prevail throughout the body, including the epidermis, digestive, reproductive, and endocrine systems.

Epithelial cells could be **simple** (stomach, intestine, or kidney), **stratified** (epidermis, esophagus, tongue), or **pseudo stratified** (remaining attached to basement membrane). Simple and pseudo-stratified epithelia consist of a single cell layer resting on a basement membrane. In contrast, stratified epithelia consist of two or more layers, with only the dividing layer of cells remaining attached to the basement membrane. Crosstalk between epithelial cells permits the tissues to respond in a coordinated manner toward the growth, differentiation, and wound healing-related stimuli. In the transformed state, keratinocytes and other epithelial cells give rise to **carcinomas** (as and when there are genetic or epigenetic alterations).

In culture, epithelial cells grow while being adhered tightly to the substrate, resulting in **flattened morphology**. The cells assort into a contiguous monolayer due to contact inhibition, the process by which monolayers of cells stop dividing and migrate once they come in contact with each other. The intermediate filament protein **keratin** is also used as a **cellular marker** for epithelial cells grown in cell culture.

5.4 Fibroblastic

These cells are elongated and could be bi- or multipolar. They grow while being attached to a substrate. Fibroblasts are typically present in almost every tissue type, originating from **embryonic mesoderm**, evidenced by the presence of intermediate filamentous protein **vimentin**. This protein, along with actin and tubulin, forms the structural support of the cell. In vivo, fibroblasts are generally wider in the middle and tapered at the ends, while remaining embedded within the connective tissue. Fibroblasts secrete proteins that are important for extracellular matrix (**ECM**) formation, including **collagen**, **elastase**, **fibronectin**, **and laminin**. These are also known to play important roles in normal physiological processes, such as wound healing through the secretion of matrix proteins, growth factors, and cytokines. In the event of injury, tissue damage stimulates fibroblasts to differentiate into myofibroblasts, which contract to enclose a wound.

These exhibit contact inhibition to keep fibroblasts from growing into monolayers when in contact with neighboring cells. Fibroblasts are also well-known for their decisive roles in many diseased states. Overproduction of connective tissue can

change its normal morphology, resulting in a state known as **fibrosis.** Transformed fibroblasts can also give rise to a cancerous form, called **sarcoma**, comprising of tumors with a mesenchymal origin. In general, fibroblasts maintain their characteristic **fusiform morphology**, but in the G/M phase of the cell cycle, they may adopt **a rounded shape** in course of preparation for cell division (mitosis and cytokinesis).

5.5 Lymphoblastic

These are immature cells that on differentiation give rise to mature lymphocytes (**B and T cells**), natural killer (**NK**) cells, and natural killer type T cells (**NKT**). They are normally found in the bone marrow, are generally **round or spherical,** and are usually **grown in suspension** without surface attachment. Lymphoblast typically modifies naïve lymphocytes (immune-responsive cells formed on account of leukocyte differentiation) with an altered morphology. It is **matured** after the activation of lymphocytes by an antigen (from **Antigen Presenting Cells**) and is increased in volume by the nucleus and cytoplasmic growth, as well as new mRNA and protein synthesis. After this stage, a lymphocyte undergoes division two to four times per 24 h for (3 to 5) days. Lymphocytes give rise to **T (T denotes Thymus) and B (B denotes Bursa Fabricius in birds) cells**, responsible for **cellular** and **humoral immunity**, respectively. T lymphocytes further differentiate into **T-helper (Th), T-suppressor (Ts), and T-cytotoxic (Tc) cells**. Helper T cells (**TH1 and TH2**) stimulate antibody production and are released in B-cells. Non-stimulated T and B cells are very similar in appearance, even when imaged using a high-resolution electron microscope. Both these cells are only slightly larger than RBCs, with very little cytoplasm being observable owing to the nucleus occupying a major cell volume.

Besides, **Neuronal Cells** have distinct morphological characteristics. They exist in different shapes and sizes but are roughly divided into two basic morphological categories: **type I** with long axons used to move signals over long distances and **type II** without axons. A typical neuron projects cellular extensions with many branches from the cell body, which is referred to as a dendritic tree. These morphological characteristics are specific to their particular role in a host. These distinctions are quite evidently observed amidst the neuron development and functional coordination in vertebrates and invertebrates.

At the early stage of cell biology research, one of the important questions that cell biologists tried to resolve is, ***"Whether Mammalian Cells/Tissue/Organs Can Be Cultured In Vitro?"***

The following paragraphs illustrate a brief interface on the basic concept of mammalian cell division, followed by cell/tissue/organ culture and the important discoveries linked to this section.

6 Concept of Increasing the Number of Cells: The Process of Cell Division

The concept of increasing the number of cells,* i.e., *cell division, is represented by three small subsections. They are as ahead:

1. A Basic Concept of Cell Division in Eukaryotes
2. Cell Cycle and Its Importance in Cell Division
3. Cell Synchronization and Its Importance in Experimental Cell Culture Research

Here is a brief discussion about them:

6.1 A Basic Concept of Cell Division in Eukaryotes

Cell division is a normal physiological phenomenon through which one somatic cell divides into two by the process of **mitosis** and one reproductive or germ cell divides into four cells initially by the process of mitosis, which is followed by **meiosis**. In humans, while in mitosis the phenotype and genotype of the mother and the two daughter cells, both are exactly similar, i.e., they contain **46** chromosomes, every germ cell, i.e., all the four germ cells contain **23** chromosomes only. Since cell division increases the number of cells, it is also called **cell proliferation**. In mammals, cell division is required not only for the **embryogenesis of a fertilized zygote** and the **morphogenesis of a growing fetus to form a complete body** (Figs. 7 and 8) but also to **replenish or** renew the old dying cells and **to repair** the damaged tissues and organs.

The role of mitosis is to replicate the genetic material in an existing cell, which is known as the **"parent cell,"** and to distribute that genetic material into two new cells, known as **"daughter cells."** The main purpose of mitosis is to accomplish cell regeneration, cell replacement, and growth in living organisms**.** Mitosis is important because **it ensures that all new cells generated in a given organism have the same number of chromosomes and genetic information**. Mitosis occurs in four discrete, consistently consecutive phases: (1) **Prophase**, (2) **Metaphase**, (3) **Anaphase**, and (4) **Telophase** (Fig. 9**)**.

6.1.1 Prophase

The first step of mitosis is called prophase. This is when the genetic fibers within the cell's nucleus, known as chromatin, begin to condense and become tightly compacted together. Once the chromatin (a mixture of chromosomes and proteins) has been condensed into individual chromosomes, the genetically identical chromosomes come together to form an "X" shape, called **sister chromatids**, which join at the **kinetochore** through **centromeres**. Slowly, spindle fiber is formed through microtubule proteins. At the **late prophase** or **early metaphase**, the nuclear membrane breaks down, leading to the commencement of the next phase.

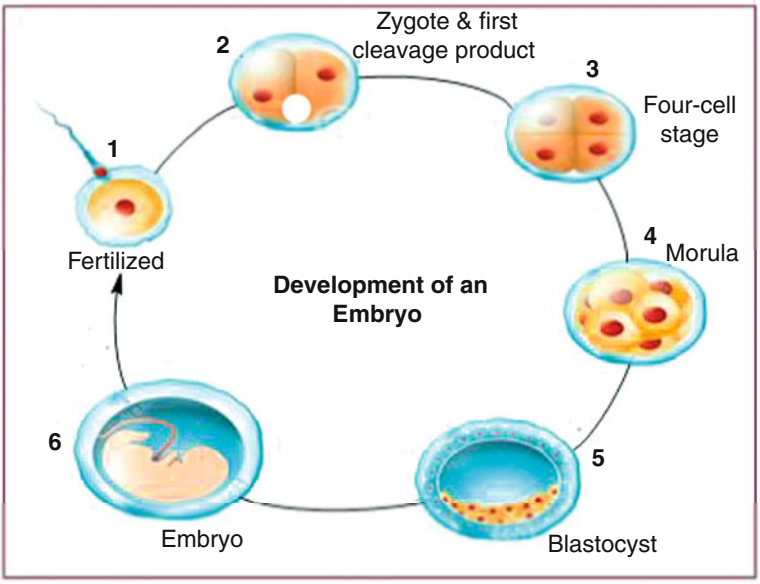

Fig. 7 Schematic stages involved in embryogenesis of a fertilized zygote. (Figure drawn by extracting inputs from https://www.shutterstock.com/image-vector/embryo-development-fertilization-zygote-morula-blastocyst-1441785692)

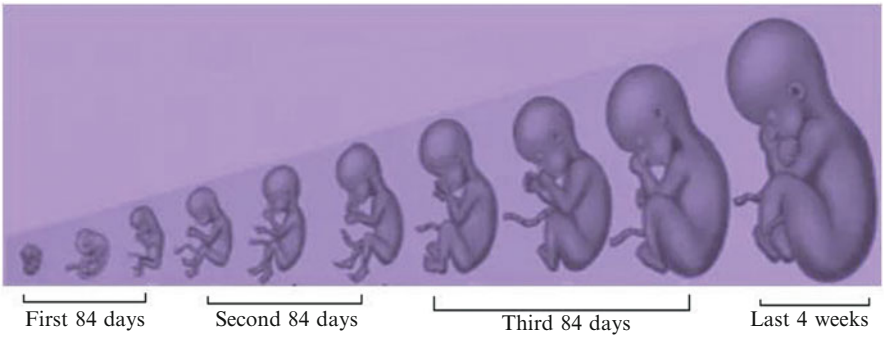

Fig. 8 Morphogenesis of a growing fetus to form a complete body. (Figure made after referring to https://www.babydestination.com/stage-wise-development-of-fetus)

6.1.2 Metaphase

Metaphase is the second step of mitosis. At the beginning of this phase (prometaphase), all the kinetochore microtubules get attached to the sister chromatids' centromeres. In the next step, the sister chromatids are equitably distributed across the metaphase plate. At this stage, the **metaphase** checkpoint screens the readiness of cells for the division which includes examining whether kinetochores are properly attached to the mitotic spindles and that the sister chromatids are **evenly distributed**

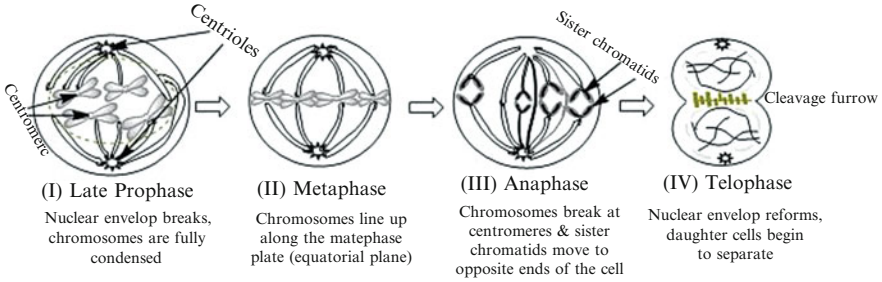

Fig. 9 Sequential stages of mitosis, responsible for maintaining a constant number of chromosomes and genetic information in all the new cells formed in an organism

and aligned across the **metaphase plate**. The positive results of the metaphase checkpoint allow the cells to move to the next phase of cell division.

6.1.3 Anaphase

Anaphase is the third phase of mitosis. It is in this phase that the two chromatids separate into two identical chromosomes and are subsequently **pulled toward the opposite ends of the cells** by the spindle fibers. This phase ensures that each chromosome receives identical copies of the parent cell's DNA. Since chromosomes are pulled at the two ends of cells, they exhibit **maximum condensation**.

6.1.4 Telophase

The **last phase** of mitosis is called telophase. This phase is characterized by the separation of telophase when the newly separated daughter chromosomes get their nuclear membranes with identical sets of chromosomes. This new nuclear envelope forms around the two sets of separated daughter chromosomes, **creating two separate nuclei inside the same cell**. Now that the two sets of daughter chromosomes are encased in a new nuclear envelope, they begin to spread out again. With the division of one nucleus into two identical nuclei, the completion of mitosis is inferred.

6.1.5 Cytokinesis

Cytokinesis is the actual division of the cell membrane, including the cytoplasm, into two discrete cells. For completing the process of cell division, cytokinesis is highly essential. To begin cytokinesis, a contractile ring (constituting protein filaments) that forms down the middle of the cell starts shrinking, pulling the cell's outer plasma membrane inwards. Eventually, the contractile ring shrinks so much that the plasma membrane pinches off and the separated nuclei can form into their cells. The end of cytokinesis signifies the termination of the M-phase of the cell cycle, of which mitosis is also a part. At the end of cytokinesis, the division phase of the cell cycle has formally ended (Alberts et al. 2002; Golitsin and Krylov 2010).

NB: While in mitosis (the division of somatic cells), one mother cell (2N) divides into two daughter cells (2N each), in meiosis (a division of germ or reproductive

cells,* e.g., *sperm and ovum), one mother cell (2N) divides into four daughter cells (each containing N number of chromosomes). This decrease in the number of chromosomes helps to maintain the number of chromosomes in offspring) as and when germ cells are fertilized (N from sperm + N from ovum = 2N).

6.2 Cell Cycle and Its Importance in Cell Division

Immediately after mitosis, when a mother cell is divided into two daughter cells, both these cells undergo a resting phase ($\mathbf{G_o}$). A G_o phase indicates that a cell is not doing anything related to cell division, including the preparation for cell division. A complex regulatory mechanism controls if and when these cells will transit from the Go phase to the preparation for cell division by the **G1 phase** (synthesis of every protein for DNA replication), **S phase** (DNA replication phase), and **G2 phase** (checking whether every material necessary for cell division is synthesized in adequate and just quantity), and finally cell division at **M phase**. This cycle of events (**Go-G1-S-G2-M**) is repeated again and again in the life cycle of a **dividing cell** (neuronal cells, mature erythrocytes, etc. are non-dividing cells and remain in the Go phase for their entire life span) as long as a cell undergoes division. While a normal cell shows a limited number of divisions (**Heyflick effect**), a cancer cell may have a very short or no Go phase and is capable of unlimited division, provided proper nutrients are supplied (Hayflick and Moorhead 1961; Rubin 1997; Wright and Shay 2002; Jafri et al. 2016).

Two classes of proteins control the regulation of the cell cycle. They are called **cyclins** and cyclin-dependent kinases (**CDKs**), a type of serine/threonine kinases. Cyclins are regulatory subunits, and CDKs are catalytic subunits of the same enzyme. Every phase of the cell cycle is regulated by specific cyclins and CDKs. For example, in mammalian cells, **cyclin D** (D1, D2, D3) regulates the G1 phase, while **cyclin E** (E1, E2) is involved in the (G1-S) phase transition, **cyclin A** (A1, A2) is predominantly involved in the S phase entry, and **cyclins B** (B1, B2, B3) are predominantly involved in G2-M transition. Each cyclin needs to bind with a specific CDK for its action. For example, in mammals, cyclin D binds with CDK4 and CDK6, cyclin E binds with CDK2, cyclin A also binds with CDK2, and cyclin B binds with CDK1. While a particular cyclin level rises during activation of a particular cell cycle phase and its level generally goes down after their activity (**hence called cyclins because** of their **cyclic appearance and disappearance**), the CDKs are maintained in their steady-state level at various cell cycle phases.

For example in the resting phase (Go), the cyclin D level of the cells is very low. As the cells enter the G1 phase, the cyclin D level increases, binding to CDK4 and CDK6 and activates the G1 phase by preparing all the proteins necessary for DNA synthesis (replication). Once the G1 phase is over, the cyclin D level again decreases due to their degradation. The cyclin D level once again increases in the next phase of the cell cycle when there is a need to replicate the DNA. That is why the cyclin name was given **(cyclic appearance and disappearance in various cell cycle phases)**.

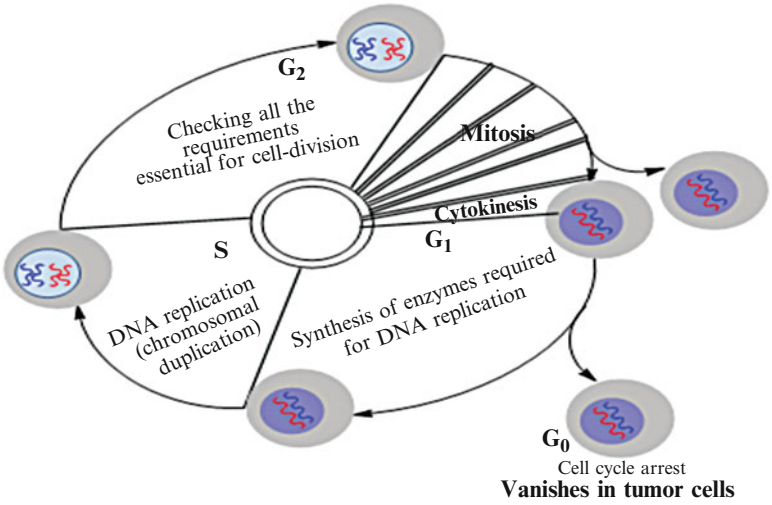

Fig. 10 Schematic description of cell-cycle stages

For experimental purposes, one can identify and subdivide a particular population of cells belonging to the G1 or S or G2 or M phase. An instrument called **a cell sorter or flow cytometer** effectively sorts out the cells based on the specific proteins that are involved in the progression of a particular cell cycle phase. In flow cytometry, antibodies conjugated with various fluorescent molecules such as **FITC, FC, or Avidin-biotin** are utilized to bind cell cycle phase-specific proteins. A flow cytometer is not only useful to cell biologists and immunologists but also to cancer biologists. As mentioned previously, cancer cells are marked by a continuous cell cycle progression without any Go phase (Morgan 2007). Figure 10 represents various cell cycle phases and their functions.

6.3 Cell Synchronization and Its Importance in Experimental Cell Culture Research

To observe the efficacy of a drug/toxin/medicinal preparation, etc. on the experimental cultured cells, **synchronization** of the cells is highly essential. Synchronization is the state in which all the cells are in a desired phase of the cell cycle. The most desired phase to stop the cell cycle is G1, since the **G1-S phase transition** is responsible for effectively initiating the cell cycle. Henceforth, the most important transition point is the **G2-M phase transition**, where cells are checked for the necessary preparations needed for cell division in adequate quantity before entering the process of cell division (mitosis/meiosis) (Harper 2005). The easiest way to stop the cells at the G1 phase of the cell cycle is **serum starvation** (withdrawal of FBS/**FCS from the cell culture medium**).

Some of the important drugs/molecules that are effective in various phases of the cell cycle are as follows:

6.3.1 Synchronization of the Cells in the G1 Phase of the Cell Cycle by Serum Starvation

Technically, at the Go phase of the cell cycle, cells are not doing anything related to cell division. Just before the commencement of the G1 phase, cells must ensure that negative inhibitory signals mediated by the tumor suppressor proteins (such as P^{53}, P^{Rb}, P^{27}, P^{21}, PTEN, etc.) are secreted at the lowest (basal) extent while the positive stimulatory signals mediated by various materials (e.g., growth factors, hormones, amino acids, etc.) are at their highest level. For in vitro mammalian cell culture, fetal calf serum (FCS) or fetal bovine serum (FBS) are added to the cell culture medium, to supply nutrients, growth factors, hormones, vitamins, minerals, etc. These molecules are necessary for synthesizing all the proteins/enzymes for DNA replication. Thus, starving cells of serum can prevent them from division. While serum starvation is the cheapest way to synchronize the cells in the G1 phase of the cell cycle, some cell lines may not be sensitive to serum starvation.

The process of serum starvation is as follows:

- Take freshly cultured cell culture containers having 60–80% confluency of exhibiting cells.
- Remove the serum-containing medium from the cell culture containers.
- Wash the cell culture containers either with Phosphate Buffer Saline (**PBS**), Hanks Balanced Salt Solution (**HBSS**), or cell culture medium without any additives, including serum.
- Add a complete serum-free medium to the culture containers.
- Incubate the cells in a CO_2 incubator for 24 to 72 h.
- Check for synchronization in the G1 phase using DNA dyes in flow cytometry.

NB: Many cells cannot tolerate the complete withdrawal of serum from the culture medium. For example, the endothelial cells perish within 18–24 h of complete serum starvation. Also, sudden/steep removal of serum from the cell culture medium may have drastic effects contrary to the step-wise gradual serum starvation.

6.3.2 A High Level of Thymidine Blocks Cells in the G1 Phase and Causes Cell Synchronization

While thymine (Dexythymidine) is one of the nucleotides needed for DNA synthesis (DNA replication), a high concentration of thymine blocks DNA synthesis. Based on this basic principle, a high thymine concentration is used to synchronize cells at the G1-S phase transition. Of note, while the G1 phase is necessary for the synthesis of all the proteins/enzymes necessary for replication, the S phase is responsible for the actual DNA synthesis.

The process of a double thymidine block is as follows:

- Seed and culture the mammalian cells in vitro.
- Allow them to grow till the mid- to early-log phase (40–50% confluency).

- Add 2 mM thymine to the culture medium and incubate overnight (~ 10–12 h).
- Wash the cells to remove excess thymine present in the medium.
- Incubate the cells with **deoxycytidine** (~ 9 h) to release them from the block.
- In simple terms, deoxycytidine restores the imbalances in nucleotide pools that occur after exposure to excess thymidine.
- Repeat the thymidine incubation.
- Release again using deoxycytidine.
- Check the blocking at the G1-S phase transition using flow cytometry.

6.3.3 Inhibition of Cyclin-Dependent Kinases Arrests the Cells at the G1 Phase

- As described in the previous paragraphs that cyclins and CDKs are two separate classes of proteins that collectively regulate various cell cycle phases. Each phase of the cell cycle is regulated by different classes of cyclins and CDKs. Collectively, while specific cyclins are involved in phase implicit cell cycle regulations, CDKs are involved in the catalytic activities.
- Based on this principle, several CDK inhibitors have been recognized that arrest the cell cycle via inhibiting the actions of various CDKs.
- For example, cyclin D (D1, D2, and D3) and CDK4/CDK6 are involved in the activation of the G1 phase of the cell cycle.
- CDK4/6 inhibitors are very effective for arresting mammalian cells in the G1 phase.
- Large pharmaceutical companies such as Pfizer, Novartis, Eli Lilly, etc. have developed several CDK inhibitors as anticancer drugs.
- Some examples of CDK inhibitors are **palbociclib** (Pfizer), **ribociclib** (Novartis), and **abemaciclib** (Eli Lilly).
- Proper optimization is needed for these CDK inhibitors' actions since some of these may have off-target effects at various concentrations (Pardee 1989).

NB: ***A large number of cells cannot tolerate serum starvation and eventually die. Similarly, thymidine block is a laborious and time-consuming technique. On the other hand, CDK inhibitors are best suited to all cells, synchronizing at a specific cell cycle phase. At present, CDK inhibitors are best suited in anti-cancer drug discovery laboratories.***

6.3.4 Synchronization of Cells in the G2 Phase of Cell Cycle

Inhibition of Microtubule Formation and G2 Arrest

Microtubules (made up of **tubulin proteins**) are one of the most important components of the **cytoskeleton** involved in cell division. They are necessary for the segregation or separation and distribution of the chromatids/chromosomes across the two ends of a dividing mother cell. Eventually, this mother cell will be divided into two daughter cells. Therefore, microtubules are very important drug targets to control the cell cycle. At present, several microtubule inhibitors are used that synchronize the cells at the G2-M transition. Experimentally, the most widely used microtubule inhibitors are **colcemid, nocodazole, paclitaxel, vincristine, and vinblastine**.

Inhibition of Cdk1 and G2 Arrest

The G2 phase of the cell cycle uses different cyclins (e.g., cyclin B) and CDKs (e.g., CDK 1). In a true sense, these cyclins and CDKs work along the G2-M phase boundary, leading to the transition of cells from the G2 to M phase of the cell cycle. The scientific community across the world has developed several CDK1 inhibitors. For example, a small molecule inhibitor, **RO-3306** (Roche), can arrest cells in the G2 phase. As indicated by the developers, the success of CDK inhibitors depends on several factors, including the cell line or cell type used, the right concentration, and optimum incubation time.

NB: A comparative scientific preference of microtubule inhibitors versus CDK 1 inhibitors claimed CDK1 inhibitors as the preferred experimental materials of interest. Most probably, this is because CDK inhibitors are generally less toxic than microtubule inhibitors, which allows the scientists to further use the cells as per the experimental requirement(s).

S Phase Synchronization

During the S phase, the replication of DNA takes place, and therefore the number of chromosomes is doubled, a prerequisite for mitosis. One of the most well-studied S phase regulators is **2[[3-(2,3-dichloro phenoxy) propyl] amino]ethanol (2,3-DCPE)**. This molecule works by activating certain **caspases** ***(a family of cysteine proteases involved in apoptosis)*** and **reducing the BCL-XL** ***(a BCL member family protein involved in antiapoptotic activities)*** **protein formation**.

6.3.5 Confirmation of Cell Synchronization

Experimental conformation mandates documentation and confirmation of cell synchronization at a specific cell cycle phase. Either **microscopy** or **flow cytometry** is used to ascertain cell synchronization at a particular cell cycle phase. Best utilization would be via phase-specific fluorescent-labeled antibodies. The protocols for microscopy or flow cytometry are discussed in their further, specialized chapters.

7 Concept of Mammalian Cell and Tissue Culture

- **Mammalian cell culture** is defined as the process by which cells are isolated from a mammalian body (specific organs or tissues) and are grown in vitro under controlled laboratory conditions of maintained **pH, temperature, humidity, O_2 tension, and readymade nutrient supply** (through cell culture medium).
- **Tissue culture** is a collective process by which whole tissues are isolated from a mammalian organ and are subsequently grown in vitro in controlled environmental conditions, either on semi-solid, solid (**aga**r), or liquid (**nutrient broth**), supplemented with nutrients and growth factors, similar to the cell culture medium.
- The term tissue culture refers to the culturing of tissue pieces or parts of an organ isolated from any plant or animal cell or organ, i.e., **explant culture**.

- The controlled laboratory conditions like pH, temperature, and humidity of the growth medium are optimized using **a CO_2 incubator**.
- **Nowadays, the term tissue culture is often used interchangeably with cell culture.**
- Different cells such as **endothelial cells, fibroblasts, smooth muscle cells, cardiac muscles, epithelial cells**, etc. present in various mammalian organs such as the **liver, breast, prostate, ovary, skin, kidney**, etc. are grown in cell culture. The specific requirement of culture medium for these cells is attained through the requisite inclusion of hormones, growth factors, specific ECM proteins, neurotransmitters, etc.
- Besides the above-mentioned, **normal mortal cells** that originated from various organs, cells from many **tumors or cancers** can also be grown in cell/tissue culture.
- Cells are taken from normal or tumor/cancer-affected tissues/organs and are generally grown as adherent cultures.
- Additionally, cells (particularly leukocytes) present in the normal or cancer-affected blood cells can also grow in **suspension culture** (Phelan 1996; Acheson 1993; Rodríguez-Hernández et al. 2014).

Here is a summary of major discoveries in the field of cell/tissue/organ culture:

8 Historical Events in the Development of Mammalian Cell Culture Technology

- Over the last 200 years, a large number of scientists have worked very hard to develop cell, tissue, and organ culture protocols.
- In early **1878**, physiologist **Claude Bernard** was the first to propose in vitro maintenance of live cells or tissues via intact physiological functions, even after an animal's death.
- In **1907**, the term "Cell Culture" was first successfully postulated by **Ross Harrison, presently remembered as the "Father of Cell Culture."**
- Mammalian cell culture became more popular in the nineteenth century after the discovery of a **chemically defined cell culture medium** by **Lewis and Lewis** for the supply of specific nutrients facilitating culturing of a particular cell type.
- At the same time, (**~1928**) invention of the **antibiotic Penicillin** by Nobel laureate **Alexander Flemming** enabled the arresting of pathogenic microbe growth (especially bacteria) in cell culture.
- **The discovery of antibiotic penicillin from *Penicillium notatum,* yeast was one of the major achievements of the contamination-free mammalian cell culture.**
- In the **mid-1900s,** with the breakthrough discovery of proteolytic enzyme **trypsin**, both cell and tissue culture emerged as essential **techniques** in modern biology laboratories. Cells that are attached to the culture containers **(adherent cells**) could now be easily detached from the surface of culture flasks for

harvesting using proteolytic enzymes such as trypsin. These harvested cells are subsequently transferred to a new culture flask with a fresh medium for **further propagation**. This technique is commonly called a **subculture** of cells.

- In terms of **two-dimensional (2D)** cell culture containers, the major discoveries were by **Julius Richard Petri (the discoverer of the Petri dish)** and **Carrel and Baker** (the discoverers of the **T flask)**.
- Other major discoveries in the mammalian cell culture were the mammalian cell culture in **three dimensions (3D),** the discovery of various instruments *(*e.g., **Bioreactors**) for the large-scale culture of mammalian cells to enable the production of various molecules such as recombinant proteins, vaccines against viruses, enzymes, etc. on an industrial scale. The basic mammalian cell culture techniques now began to be used for **organ, organotypic and histotypic cultures.**
- Besides various **human cells (originated either from healthy or diseased organs such as tumors and cancers)**, both **embryonic and adult mouse cells** (being an inexpensive and reproducible source of biological experiments) could be used for mammalian cell culture regularly. Other mammalian cells that originated from **rats, monkeys, or guinea pigs** also emerged as a provision for large-scale cell culturing (Dumont et al. 2016; Jedrzejczak-Silick 2017).
- ***Here is a chronological list of events as the major discoveries in the field of cell/tissue/organ culture:***
- **1595**: ***Zacharias Janssen*** *and his father* ***Hans*** is credited with the invention of the first compound microscope.
- **1655**: ***Robert Hooke*** described "**cells**" in **cork**.
- **1674–1676**: ***Anton Van Leeuwenhoek*** discovered protozoa. He observed bacteria some 9 years later.
- **1833**: ***Robert Brown*** described the cell **nucleus** in orchid cells.
- **1838**: ***Schleiden*** *and* ***Schwann*** proposed **cell theory**.
- **1840**: ***Albrecht von Roelliker*** realized that sperm and egg are nothing but cells.
- **1856**: ***Pringsheim N***. observed how a sperm cell penetrated an egg.
- **1855–1858**: ***Rudolf Virchow*** (physician, pathologist, and anthropologist) expounds his famous conclusion: *Omniscellulaecellula*, that is **cells develop only from pre-existing cells** [cells come from pre-existing cells].
- **1857**: ***Albert von Kolliker*** described **mitochondria for the first time (*Carl Benda*, in 1898, used the term mitochondria).**
- **1877: *Julius Richard Petri*** discovered Petri Dish.
- **1878: *Claude Bernard*** proposed that the physiological systems of an organism can be maintained in a living state even after the organism's death.
- **1880: *Amoled*** showed that leukocytes can divide outside the body.
- **1882: *Walter Flemming*** discovered the process of cell division.
- **1885: *Wilhelm Roux*** illustrated the maintenance of embryonic chick cells in a saline culture.
- **1897: *Loeb*** demonstrated the survival of cells isolated from blood and connective tissue in serum and plasma.
- **1898: *Camillo Golgi*** described the Golgi apparatus.

- **1898:** ***Liunggren*** for the first time maintained human tissue (skin) in vitro in the ascetic fluid.
- **Nineteenth century:** ***Sydney Ringer*** developed salt solutions containing the chlorides of sodium, potassium, calcium, and magnesium, suitable for maintaining the functioning of an isolated animal heart outside the body.
- **1903:** ***Jolly*** studied the behavior of animal cells immersed in serum lymph (**Also observed cell division of salamander leukocyte)**.
- **1907:** ***Ross Granville Harisson*** cultivated frog nerve cells in lymph clots and observed the growth of nerve fiber in vitro. He is considered then **"Father of cell culture."**
- **1910:** ***Burrows*** succeeded in the long-term cultivation of chicken embryo cells using the **plasma clot method**. He made detailed observations of mitosis.
- **1911*: Lewis and Lewis*** made the **first liquid cell culture medium** consisting of sea water, serum, embryo extract, salts, and peptones. They observed limited monolayer growth.
- **1912–1913:** ***Carrel*** introduced strict aseptic techniques so that cells could be cultured for long periods.
- **1916:** ***Rous and Jones*** introduced proteolytic enzyme **trypsin** for the subculture of adherent cells.
- **1920:** The ***ECACC*** is established for cell culture preservation.
- **1922:** ***Albert Ebeling*** cultured epithelial cells for the first time.
- **1923:** ***Carrel and Baker*** developed "Carrel" or **T-flask** as the first specifically designed cell culture vessel. They studied microscopic observations of cells in culture.
- **1923:** ***Carrel and Ebeling*** performed a subculture of the fibroblastic cell line.
- **1924:** ***Levitsky for the first time,*** used the term **karyotyping**.
- **1925:** The ***ATCC*** is established for cell culture technique evaluation.
- **1925–1926**: ***Strangeweys and Fell*** discovered cell differentiation through in vitro organ culture.
- **1926:** ***Svedberg*** developed the first analytical ultracentrifuge.
- **1920–1930:** ***Carrel and Ebeling*** **subcultured the fibroblast cell line for the first time**.
- **1927:** ***Carrel and Rivera*** produced the first viral vaccine: Vaccinia.
- **1928:** ***Alexander Flemming*** discovered penicillin from *Penicillium notatum.*
- **1930:** ***Carrel and Linberg's*** new cell culture devices discovery.
- **1933:** ***George Gey*** developed the **roller tube technique** for cell culture.
- **1938:** ***Behrens*** used differential centrifugation to separate nuclei from the cytoplasm.
- **1943:** ***Earle*** *and coworkers* established the L-cell mouse fibroblast cell line, the first-ever continuous cell line.
- **1948:** ***Keilova, Cruikshank, and Lowbury*** introduced **antibiotics** in cell culture.
- **1948:** ***Sanford*** derived clone 929 from L cell line.
- **1948:** ***Fischer*** developed a chemically defined medium, **CMRL 1066.**
- **1949:** ***Enders*** established the growth of a virus in cell culture.

- **1951–1952:** ***George Gey*** cultured continuous human cancer cell line (**HeLa**) from human cervical carcinoma for the first time.
- **1952:** ***Kew*** *and coworkers* reported ***polio virus*** culturing for the first time in monkey kidney cells.
- **1952:** ***Dulbecco*** developed a plaque assay for animal viruses using cultured cell confluent monolayers.
- **1953:** ***Crick, Wilkins, and Watson*** proposed a **double-helical DNA structure**.
- **1954:** ***Abercrombie*** observed contact inhibition through ceased motility of diploid cells in monolayer culture upon establishing contact with adjacent cells.
- **1955:** ***Eagle*** studied the nutrient requirements of selected culture cells and established the first widely used **chemically defined medium**.
- **1856–1857:** ***Louis Pasteur*** **for the first time showed that lactic acid fermentation is caused by a living organism**.
- **1957:** ***Meselson, Stahl,*** *and* ***Vinograd*** developed density gradient centrifugation in cesium chloride solutions for separating the nucleic acids.
- **1958:** ***Coriell*** recognized the role of mycoplasma in cell culture contamination.
- **1959:** ***Puck Marcus*** conducted the cloning of HeLa cells on a homologous feeder layer.
- **1961:** ***Sorieul*** *and* ***Ephrussi*** discovered cell fusion and s**omatic cell hybridization**.
- **1961:** ***Hayflick*** *and* ***Moorhead*** isolated human fibroblasts (WI-38), demonstrating their finite lifespan in culture, famously known as the **Hayflick** effect.
- **1962:** ***Macpherson*** *and* ***Stoker***'s establishment and transformation of BHK21 cells.
- **1964:** ***Klein Smith*** *and* ***Pierce*** discovered the pluripotency of embryonic stem cells.
- **1964:** ***Littlefield*** introduced the HAT medium for cell selection.
- **1965:** ***Ham*** introduced the **serum-free medium**, which was able to support the growth of some cells. Discovered serum-free cloning of **Chinese Hamster Ovary** (**CHO**) cells.
- **1965:** ***Harris*** *and* ***Watkins*** were able to fuse human and mouse cells using a virus.
- **1967:** ***Hoover*** *and* ***Cohen*** discovered epidermal growth factor (**EGF**).
- **1968:** ***Stoker*** *and coworkers* established anchorage-independent **microbial growth**.
- **1969:** ***Metcalf*** discovered colony formation in hematopoietic cells.
- **1970:** ***Kruse*** *and coworkers* developed the first-ever **laminar flow cabinets for cell culture**.
- **1975:** ***Kohler*** *and* ***Milstein*** produced the first **hybridoma cells** capable of secreting a **monoclonal antibody.**
- **1976:** ***llmensee*** *and* ***Mintz*** established the **totipotency** (the capacity to proliferate and differentiate into a complete body) of embryonic stem cells.
- **1976:** ***Hayashi*** *and* ***Sato*** prepared growth factor-supplemented **serum-free medium** for the first time.

- **1976:** ***Sato*** *and colleagues* published papers showing that different cell lines require different proportions of hormones and growth factors in serum-free media.
- **1977:** ***Nelson-Rees*** *and* ***Flandermeyer*** confirmed cross-contamination of many cells through HeLa cells.
- **1978:** ***Ham*** *and* ***McKeehan*** prepared MCDB selective serum-free medium.
- **1978: Sato** established the basis for the development of serum-free media from cocktails of hormones and growth factors.
- **1978:** ***Prindull G.*** *and coworkers* (***N. Van Der Meulen***) discovered stem cells in human cord blood.
- **1980–1987:** ***Peehl*** *and* ***Ham, Hammond,*** *and* ***Kendler*** developed many specialized cell lines.
- **1981:** ***Martin*** *and coworkers* developed in vitro stem cell lines from mice for the first time.
- **1982:** ***Genentech*** licensed human insulin as a recombinant protein for therapeutic purposes for the first time.
- **1982: Kleinman H.K**. *and others* first **described the use of matrigel and other materials for 3D culture.**
- **1983:** ***Bell*** *and others* developed reconstituted cell cultures.
- **1984:** ***Collen*** illustrated the development of recombinant tissue-type plasminogen activator in mammalian cells.
- **1983–1985:** ***Genentech*** produced human growth hormone from recombinant bacteria, subsequently accepted for therapeutic use.
- **1986:** Licensing of Lymphoblastoid γ-IFN (gamma interferon).
- **1987:** Doctors ***Mario R. Capecchi, Martin J. Evans, and Oliver Smithies*** **created the first-ever knockout mouse.**
- **1987:** Commercial availability of tissue-type plasminogen activator (tPA) from recombinant animal cells.
- **1989:** ***Weinberg*** explained **oncogenes, malignancy, and transformation.**
- **1989:** ***Kleinman KS*** and coworkers used recombinant erythropoietin protein on clinical patients (a clinical trial) for the first time.
- **1990:** Recombinant products in a clinical trial (HBsAG, factor VIII, HIVgp120, CD4, GM-CSF, EGF, mAbs, IL-2), were postulated.
- **1992:** ***SkinEthics*** produced human tissue and neural stem cell cultures in vitro.
- **1995:** ***Thomsom JA*** *and coworkers* developed an embryonic stem cell line from a primate for the first time.
- **1996:** ***Willmut*** *and coworkers* created the first **transgenic sheep, Dolly,** through the nuclear transfer technique (announced in 1997).
- **1997:** PPL Therapeutics produced a transgenic **lamb, Polly.**
- **1998:** ***Teruhiko Wakayama*** and coworkers cloned mice from somatic cells.
- **1998:** ***Aigner*** produced cartilage using tissue-engineered cell culture.
- **1998:** ***Thomson and Gearhart*** isolated and cultured the human embryonic stem cells.
- **1999**: ***Hamilton*** *and* ***Baulcombe*** discovered **si-RNA** as part of post-transcriptional gene silencing (PTGS) in plants.

- **2000:** ***Dennis*** *and coworkers* formulated a **human genome project** along with genomics, proteomics, genetic deficiency, and expression errors.
- **2000:** ***Keril*** established a robust cell-based system for genetic and functional analysis of HCV replication in culture.
- **2000:** ***Kwon*** established the rapid cell sheet detachment from **polyisopropyl acrylamide-grafted porous cell culture membranes**.
- **2000:** ***Benjamin*** described the derivation of **ES cells** from human blastocysts for somatic differentiation in vitro.
- **2001:** ***Edna*** *and others* described the 3D matrix composition and function of adhesion, derived from tissue or cell culture.
- **2001:** ***Nicole*** *and others* showed enhanced hepatitis C virus RNA replication by cell-culture adaptive mutations.
- **2001:** ***Chu*** *and* ***Robinson*** established industrial choices of protein production by large-scale **cell culture** using standard **stirred tank reactors**.
- **2001:** ***Potter*** *and* ***DeMarse*** developed a new method for cell culturing that maintains their health and sterility for many months.
- **2001:** ***Keefer*** *and others* **cloned goats** by nuclear transfer of **adult somatic cells**.
- **2002:** ***Brinster*** developed the testis cell transplantation method, providing a powerful approach to studying the biology of the male germline stem cells and their specific environment via stem cell niche.
- **2002:** Human Genome Society of France claimed the production of a **cloned human baby**, named Eve (Clonaid).
- **2002:** ***Shao*** *and others* described the SILAC method for stable isotope labeling of amino acids in cell culture for in vitro incorporation of specific amino acids into all mammalian proteins.
- **2002:** ***Atala and Lonza*** exploit tissue engineering.
- **2003:** ***Park*** *and* ***Shuler*** integrated cell culture with **micro-fabrication technology**.
- **2004:** ***Cowan*** and colleagues showed that the adipose tissue-derived adult stromal cells healed critical-sized mouse calvarial defects and skeletal defects without genetic manipulation or the addition of exogenous growth factors.
- **2004:** ***Kunz Schughart*** *and colleagues* discovered the use of **3D cell culture** for high throughput screening: **the multicellular** spheroid model detects specific cellular effects reflecting actions on various targets.
- **2004:** ***Sin*** *and coworkers* developed the three chambers of **micro-scale cell culture analog device** with integrated dissolved oxygen sensors. The method provides an in vitro supplement to animal studies with a possibility of a human surrogate for predicting human response in clinical trials.
- **2005:** ***Fulcher*** *and coworkers* described for the first time the culture of well-differentiated human airway epithelial cells.
- **2005:** ***Jonathan*** *and others* described the computer-controlled **microcirculatory support system** for endothelial cell culture.
- **2005:** ***Wang*** *and colleagues* postulated the **application of bioreactors as** powerful tools for large-scale mammalian cell culture.
- **2005:** ***Xu*** *and others* discovered **inkjet printing** of viable mammalian cells.

- **2005:** ***Yaakov*** *and others* discovered laser-guided **direct writing for 3D tissue engineering**, with underlying processes such as liver and pancreas morphogenesis, differentiation, and angiogenesis.
- **2006:** ***Shah*** *and colleagues* discovered **nucleo-counter**, an efficient technique for determination of the cell number and viability in the animal cell culture process.
- **2006:** ***Yamanaka*** obtains induced pluripotent stem cells.
- **2007:** ***Pampaloni*** *and others* described that 3D culturing bridges the gap between cell culture and live tissues. They illustrated a strong impact of 3D culture on drug screening and the use of laboratory animals for experimental purposes.
- **2007:** ***Yu*** *and coworkers* used viral vectors to reprogram adult cells to an embryonic state (**induced pluripotent stem cells**).
- **2007:** ***Parker*** *and* ***Townley*** described the biomimetic properties of **photonic nanostructures** in cell culture.
- **2008***:* ***Smalley*** *and colleagues* described a 3D cell culture model for the first time.
- **2008** ***and beyond***- Era of induced pluripotent stem cells in therapy-ongoing promises and challenges.
- **2009:** ***Fernandes*** *and others* described the scale-up of human embryonic stem cell culture in a stirred **micro-carrier system**.
- **2010:** ***Barbulovic-Nad*** *and colleagues* described a **microfluidic platform** for complete mammalian cell culture – the automated cell micro-culture technique.
- **2010+** ***Atala demonstrates*** 3D bioprinting techniques for tissue and organs.
- **2013:** ***Castro*** *and others* illustrated the high-throughput single nucleotide polymorphism (**SNP)-based authentication of human cell lines** (a solution for misidentification of cell lines).
- **2013:** ***Dayeh*** *and others* proposed the use of fish-derived cell lines for the determination of environmental contaminants.
- **2013:** ***Kalos*** *and* ***June*** **proposed the adoptive T cell transfer for cancer immunotherapy** in the synthetic biology era.
- **2013:** ***Kanatsu Shinohara*** *and* ***Takash*** discovered spermatogonial stem cell renewal and development, enabling the use of these cells for application in animal transgenesis and medicine.
- **2013:** ***Lee*** *and colleagues* described the photostable fluorescent organic dot with aggregation-induced emission for noninvasive long-term tracing.
- **2014:** ***Landuer*** designed the medium for industrial-scale CHO cell culture.
- **2015:** ***Takahashi*** described the gene transfer into cultured mammalian embryos through electroporation.

9 Selection and Validation of Appropriate Mammalian Cells for Culture and Experiments

- As discussed in the earlier paragraphs, the mammalian body is made up of two types of cells, i.e., somatic cells and germ or reproductive cells. In a mammalian body, all the somatic cells are genetically the same.

- It was also discussed previously that within a cell at a particular time, while certain genes are **transcriptionally switched on**, others may be **transcriptionally switched off**. This switching on or switching off of genes (**transcriptional regulation**) in somatic cells differs from one cell type to another.
- Thus, **due to differential expression** patterns, **mRNAs** and **proteins** expressed by one type of somatic cell may vary in another type of somatic cell. Changes in the protein expression patterns lead to **structural (phenotypic)** and **functional** variations from one another type of somatic cells.
- So, every somatic cell has its own structure and functions. For example, neuronal cells look like a tree and are involved in nerve conduction, and smooth muscle cells look like a spindle and are involved in involuntary muscle contraction.
- All cell lines, whether normal (having a definite life span) or immortal/transformed (growing continuously), originate from a normal mammalian cell.
- All the cell lines, whether normal/mortal or immortal (tumor/cancer) can be characterized based on their origin, except **anaplastic cells**, which change vibrantly (a high level of mutation occurs in these cells), it is difficult to identify their originating cells.

Thus, it validates and necessitates selecting or choosing a particular type of somatic cell or cell line for the very purpose of culturing and examining a particular structure/function related to this particular cell line.

- A large number of databases are nowadays available to wisely select the particular cell line for culture and research (Amirkia and Qiubao 2012).

The selection and validation of an appropriate mammalian cell line can be explained by discussing the following points:

1. Process of Selection of Appropriate Cells for Culture
2. Validity and Authenticity of the Chosen Cells
3. Importance of Working with Low Passage Number Cells
4. Examine Whether the Chosen Cells Are Contamination Free
5. Examine Whether the Chosen Cells Exhibit the Right Biology
6. Examine Whether the Chosen Cells Are Suitable for the Desired Experiments

Here is a brief explanation of the above points:

9.1 Process of Selection of Appropriate Cells for Culture

- At the outset, we need to understand why we are culturing mammalian cells. For example, if we are examining the expression and function of a general protein (such as glyceraldehyde-3-phosphate dehydrogenase, or GAPDH, a protein involved in glycolysis) for basic research, we can choose any mammalian cell

for culture. This is because GAPDH is a glycolytic enzyme and is expressed by all metabolic cells of a mammalian body.

- If our purpose is to produce a large amount of a particular protein for industrial purposes, we need to check what kind of cell may be suitable for our culture. For example, **CHO or HEK-293 cells** are extensively used in the **bioprocessing industry** for the production of various proteins or enzymes because of their specific capacity for mammalian-specific post-translational modifications, particularly glycosylation of proteins.
- If our purpose is to monitor the responsive behavior of cancer cells under some specific biochemical conditions, we urgently require the selection of a cancer cell in a culture medium that may be best fitted to our interest.
- If our purpose is more specific, for example, to check the role of estrogen on breast cancer complications, we must select a breast cancer cell line (e.g., **MCF-7 cell line**) that expresses estrogen receptors **(ERs**) and then the culture or treat them with various estrogens.

To confirm whether the cell line we have chosen is suitable for our experiment or not, we need to compare the biomarker expression and its specific expression extent through the literature survey.

9.2 Validity and Authenticity of the Chosen Cells

- Working with more than one cell line simultaneously may accidentally **cross-contaminate** one cell with the other. For example, several reports suggest that **HeLa cells** can easily contaminate other cells if cultured at the same time. Isolated from aggressive cervical adenocarcinoma, these cells have emerged as one of the most probable contaminants for more than the last 50 to 70 years. Studies voiced such contaminations through DNA **fingerprinting of short tandem repeats (STRs)** at selected locations, wherein one investigation noticed an indistinguishable fingerprint of the human endometrial cell line, HES, and the HeLa cells. Reports from international cell banks reveal that cell line improper identification was 16% in 1977 and increased to 18% in 1988, suggesting the persistence of this problem over a decade. Although HeLa cells remain major contamination suspects, the menace of contamination is not limited to them, if the observations from ATCC are to be believed. The data bank claims cross-contamination of continuous cell lines to be a pervasive issue in biomedical research, wherein one instance found that a human epithelial cell line immortalized by human telomerase reverse transcriptase overexpression is nothing but the human breast cancer cell line, MCF-7. The importance of this cross-contamination has been a menace, and it was only this troubling bottleneck that laid the basis for cell line authentication through the introduction of **genetic markers by *Gartler* in 1967** (Gartler 1967).
- Henceforth, many variations include inspection of banded marker chromosomes and visualization of chromosomal pattern and architecture, Human Leucocyte

Antigen (**HLA) typing**, enzyme polymorphisms, DNA polymorphisms, and relatively recent, DNA fingerprinting and use of locus-specific probes (Lee 1991).

- Another problem of working with more than one cell line is **labeling mistakes** during cell culture and experiments.
- Further, during storing of cells in an ultra-cold (liquid nitrogen) environment, **labeling may be removed** as there are significant chances of misidentification.
- The database is now available for **"Cross-contaminated or Misidentified Cell Lines."** Here is one of the important web pages:
  http://standards.atcc.org/kwspub/home/the_international_cell_line_authentication_committee-iclac_/Database_of_Cross_Contaminated_or_Misidentified_Cell_Lines.pdf

So, authentication of the mammalian cell line is indeed a crucial step in mammalian cell culture.

- It is claimed that cell banks like the American Type Culture Collection (**ATCC**), the German Collection of Microorganisms and Cell Cultures/**DSMZ**, the European Collection of Cell Cultures (**ECACC**), the Japanese Collection of Research Bio-resources (**JCRB**), the RIKEN Bio-resource Center Cell Bank/ **RIKEN** properly **authenticate** and **quality control** the cell line(s) before sending it/them to the probable buyer (Hay 1992).
- Many methods, such as **isoenzyme analysis, karyotyping, human lymphocyte antigen (HLA) typing,** and characterization of **amplified fragment length polymorphisms (AFLP),** have been used to identify cross-contamination in cell culture.
- However, a superior method is **Short Tandem Repeat (STR)** profiling, which is well-established for identification and confirmation of a mammalian cell line and DNA-based forensic identification. STR loci consist of short, repetitive sequence elements (3–7) base pairs in length. STR may have the following repetitive sequences: **Pentanucleotide:** AAAAGA AAAGAAAAGAAAAGA; **Tetranucleotide:** AAAG AAAGAAAGAAAG; **Trinucleotide:** CTT CTTCT TCTTCTT; **Dinucleotide:** AG AGAGAGAGAG (Master et al. 2001).
- The number of repeats in STR loci can be highly variable among individuals, which makes these genetic markers effective for **human identification** or **cell line authentication purposes**. STRs have become **popular DNA repeat markers** because they are easily amplified by the polymerase chain reaction **(PCR)** using PCR primers that bind to the flanking regions surrounding the STR repeat. Multiple STR loci can be examined simultaneously to create a DNA profile. Generally, **Multiplex Real-time PCR is the best method to confirm STR** (Cabrera et al. 2006; Liscovitch and Ravid 2006; Cooper et al. 2007).

9.3 Importance of Working in Low Passage Number Cells

- When a normal (mortal) cell is cultured again and again through several passaging, it will start losing many of its characteristics due to **aging/senescence**.

Finally, every mortal cell must lose the capacity of the division after a certain number of divisions and ultimately perishes ***(Heyflick effect)***.

- For example, after 4 to 5 passages, primary cultured endothelial cells start losing estrogen receptors, and after around 15 passages, these cells lose the majority of their estrogen receptors.
- Although, in general, endothelial cells have the capacity of around **50 generations**.
- Another important problem in the continuous passaging of mammalian cells is the genetic drift leading to the alteration of several changes in the growing cell line.
- Not only normal cells but even **some cancer cells** exhibit genetic drifting. Before culturing, one must check instructions from the supplier (e.g., **ATCC**), such as how many continuous passages of a cell line are possible without any genetic drifting.
- Therefore, if somebody's purpose is to check the effect of estrogens on primary cultured endothelial cells, we must choose early passaged primary cultured endothelial cells. If our purpose is to ascertain the cell proliferation and cell cycle, the cancer cell may be chosen because most cancer **cells have** no **or short G_o phase that leads to continuous division and accumulation** of a large **number of cells in a relatively short duration.**

9.4 Examine Whether the Chosen Cells Are Contamination-Free

- The serum is one of the growth-promoting agents used in mammalian cell culture. For mammalian cell culture (depending upon the type of cells), 5–20% of fetal calf serum (**FCS**) or fetal bovine serum (**FBS**) is generally used. **The serum is the most prominent source of contamination with mycoplasma, which cannot be removed by filtration sterilization because of its small size (mycoplasma is claimed to be the smallest living creature on this planet earth)**. **Trusted cell sources will supply serum/cell stocks as verified mycoplasma-free** (McGarrity et al. 1984; Lincoln and Gabridge 1998).
- Additionally, during routine culture or subculture, cells may get contaminated with **bacteria, fungus,** and other microorganisms that are present in the environment (**air/water/floors of the laboratory/on the instruments**, **virtually everywhere**). The contaminated microorganism can spoil the cell culture because of its relatively **short doubling time (e.g., *E coli* may have a doubling time of just 20 min)**. Various pathogenic viruses are other sources of contamination, particularly cell biologists should be aware of the genetically transmitted viruses (Weiss 1978).
- If the contamination status of a cell stock is not known, it is good practice to begin culturing in a **quarantine environment** and establish a **"clean" status** before moving into general circulation. Therefore, every effort should be made to get a contamination-free culture. Chapter of this book entitled, ► "Microbial Contamination of Mammalian Cell Culture" separately discusses the contamination-associated problems in mammalian cell culture.

9.5 Examine Whether the Chosen Cells Exhibit the Right Biology

- **The Cancer Line Encyclopedia:** A chosen cell line must fulfill its biological functions as per the knowledge acquired from the literature. For example, the **cancer line encyclopedia (CCLE**) represents around 1000 cancer cell lines. This representation includes the basic knowledge about various established cancer cell lines, their origin, specific genetic aberrations (mutations) related to various cell lines, markers to identify a specific cell line, and so on. These details help the researchers to select specific cell lines of interest.
- **The Catalogue of Somatic Mutations in Cancer: The catalogue of Somatic Mutations in Cancer (COSMIC)** is an excellent resource for somatic mutations of several cancer cell lines.
- Besides applied biology such as cancer, one may choose to work on basic cell biology and physiology. So, based on the specific need, cell lines must be selected. For example, endothelial cell lines, as well as primary cultured endothelial cells would be the specific choice of interest if somebody intends to work on both, the physiology of vasomotion as well as the pathophysiology of atherosclerosis.

9.6 Examine Whether the Chosen Cells Are Suitable for Desired Experiments

- Every cell line has its morphology (**phenotype**) and a few characteristic biological activities. Morphology and biological functions depend on the cell culture medium composition and culture conditions, which again influence the growth rate, transfection ability (e.g., strictly adherent cells such as endothelial cells exhibit a low transfection rate), and other cellular characteristics. Thus, several factors influence the selection of cell lines for specific experimental purposes.
- Cost-effectiveness (for handling, maintenance, and experimental use) is one of the most important factors that may influence the selection of a particular cell line.
- For example, while induced pluripotent stem cells (**iPSCs**) may represent a more "normal" model than a cancer cell line, the culture and maintenance costs of iPSCs prevent their use in some laboratories.

NB: American Type Culture Collection (ATCC) has the world's largest and most extensive database of various cell lines, including more than 4000 continuous cell lines. The database includes species, the tissue/organ-specific origin of the cell lines, and specific signaling pathways involved in both physiology and pathophysiology.

10 Conclusions

This introductory first chapter describes the process of formation of the mammalian body through **cells**, **tissues, organs, and organ systems**. This chapter primarily focuses on **cell theory,** besides the identification and characterization of cells via karyotyping and cell morphology. As the chapter goes forward, the process of mammalian **cell division,** i.e., mitosis and meiosis are discussed very briefly. The topic of cell division is addressed, as cell division is the fundamental key process for increasing the number of cells or cell proliferation. Finally, the main topic of this introductory chapter, i.e., the basic concept of mammalian cell culture, has been discussed. Once again, **mammalian cell culture** is defined as the process by which cells are isolated from mammalian tissues or organs and are grown in vitro in readymade synthetic nutrients under controlled laboratory conditions of maintained pH, temperature, and humidity in either a CO_2 incubator or in an industrial-scale Bioreactor. Today, **the terms cell culture and tissue culture are often used interchangeably**. To understand the various aspects of cellular physiology, particularly the effects of various drugs/toxins, etc. on the cultured cells, **synchronization** of different cultured cells is highly essential. Synchronization is a particular state in which all the cells remain simultaneously in the desired phase (**G1-S-G2-M**) of the **cell cycle.** The most desired phase to stop the cell cycle is the G1 phase since the G1-S transition is responsible for effectively initiating the cell cycle. The easiest way to stop the cells at the G1 phase of the cell cycle is **serum starvation**. As an introductory chapter, age-old discoveries of various technological aspects of mammalian cell culture by different scientists are mentioned carefully. The chapter finally addresses the process of validation and authentication of chosen mammalian cells. This section of the chapter mainly addresses six major points, i.e., (1) Process of selecting appropriate cells for the initiation of culture; (2) Validity and authenticity of the chosen cells; (3) Importance of working with the cells having a low passage number; (4) Examine and monitor any microbial or chemical contamination in the chosen cells; (5) Confirm whether the chosen cells exhibit the right morphology and physiology; (6) Determine whether the chosen cells are suitable for desired experiments**.** The chapter ends with the identification and validation of the chosen cells before commencing the cell culture.

11 Cross-References

- ▶ Bioimaging: Usefulness in Modern Day Research
- ▶ Biosensors' Utility in Mammalian Cell Culturing
- ▶ Common Reagents and Medium for Mammalian Cell Culture
- ▶ Culture of Continuous Cell Lines
- ▶ Culture of Neuron and Glia Cells
- ▶ Emerging Drug Delivery Potential of Gold and Silver Nanoparticles to Lung and Breast Cancers
- ▶ Establishment of a Cell Culture Laboratory

- Experimental Mammalian Cell Culture-Based Assays
- Isolation and Purification of Various Mammalian Cells: Single Cell Isolation
- Isolation and Primary Culture of Various Mammalian Cells
- Large-Scale Culture of Mammalian Cells for Various Industrial Purposes
- Mammalian Cell Culture in Three Dimensions: Basic Guidelines
- Mammalian Cell Culture Laboratory: Equipment and Other Materials
- Mammalian Cell Culture Types and Guidelines of Their Maintenance
- Mammalian Cells: Reliability as Model System in the Ecotoxicological Evaluation of Environmental Stressors
- Mammalian Cells, Tissues and Organ Culture: Applications
- Microbial Contamination of Mammalian Cell Culture
- Nanomaterials: Compatibility Towards Biological Interactions
- Organ, Histotypic and Organotypic Culture, and Tissue Engineering
- Primary Culture of Immunological Cells
- Stem Cell Culture and Its Applications
- Troubleshooting of Mammalian Cell Culture

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Establishment of a Cell Culture Laboratory

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https://doi.org/10.1007/978-981-19-1731-8_2-1

Abstract

The present chapter describes the **design of a mammalian cell culture laboratory** equipped for scientific research purposes. In addition to the main cell culture facility (containing sterile laminar flow hood, CO_2 incubator, inverted microscopes, etc. for safe transfer and culture of mammalian cells) and anti-room (to prevent direct microbial contamination from outside aerial flow), the outer laboratory should be divided into the following areas: (1) a general washing area; (2) a medium preparation, sterilization, and storage area; and (3) an observation/data collection area. For the design of a mammalian cell culture laboratory, **biosafety** is the most important aspect which is addressed meticulously. Starting from minimum to maximum biohazard containment, four biosafety levels, chronologically sequenced as 1–4, are described. Another crucial aspect in designing a mammalian cell culture laboratory is the **care and maintenance of the laboratory area** since microbial **contamination** is a major problem. Finally, the **safety of both the laboratory personnel and the cell culture laboratory itself** needs to be addressed. The safety of a mammalian cell culture laboratory can be maintained by the following important instruments: (1) s**afety instruments specific for mammalian cell culture** (laminar flow hood containing HEPA filters, UV light, and CO_2 incubator) and (2) **safety instruments common to any research laboratory** (first aid kits, chemical spill kits, fire extinguishers, fire blankets, chemical fume hoods, safety showers, eyewash stations, refrigeration equipment, flammable liquid storage cabinet, safety cans, and portable safety shield). *All necessary sterile and safety equipment needed to develop a mammalian cell culture laboratory are discussed in this chapter.*

Keywords

Mammalian Cell Culture Laboratory Setting Up · Cell Culture Instruments · Biosafety Levels (Level 1, Level 2, Level 3, Level 4) · Biosafety Instruments · Cell Culture Laboratory Care and Maintenance

1 Introduction

A proper design of a mammalian cell culture laboratory is a prerequisite for the successful culture of mammalian cells. The present chapter describes the design to set up a mammalian cell culture laboratory for routine laboratory research. There needs to be necessarily a provision for a general washing area, sterilization, medium preparation and storage area, an anti-room, an aseptic transfer area, the main cell culture facility, and finally an observation/data collection area. All these areas collectively form a complete mammalian cell culture laboratory.

Biosafety is one of the important areas that need to be considered for an aseptic and viable mammalian cell culture. Based on the type of biohazards, material biosafety levels can be changed or enhanced. Starting from minimum to maximum biohazards, four biosafety levels, i.e., biosafety levels 1–4 are designated. Besides biosafety levels, environmental factors encompass multiple important roles in the laboratory-scale culture and growth of mammalian cells. In general, for the ideal culture of mammalian cells, a temperature of 37 °C, CO_2 level of 5%, moisture content of 95%, and medium pH of around 7.4 are required.

One of the major problems of mammalian cell culture is **contamination** with various microorganisms. Microorganisms can be present anywhere in the laboratory environment including air, water, the laboratory floor, or even on the surfaces of various instruments. Microorganisms may be present in our hands also. Since microorganisms have a very short doubling time (e.g., bacteria have a doubling time of just 20–30 min) and therefore if microorganisms contaminate the cell culture medium, they will rapidly proliferate, completely use up the cell culture medium, and overwhelm the slowly growing mammalian cells. Therefore, cleaning and maintaining the mammalian cell culture laboratory area are of utmost importance. While a separate chapter (chapter ▶ "Microbial Contamination of Mammalian Cell Culture") is dedicated to understanding various microorganisms, contamination mediated by these, and its prevention, this chapter briefly describes various precautionary steps that reduce the chances of contamination.

Finally, safety is the most important factor of working in any kind of research laboratory that includes not only the personnel working in the mammalian cell culture laboratory but also the laboratory premises. People working in the laboratory may be exposed to various biological and chemically hazardous materials. Besides, like every other laboratory, accidents (such as fire incidents) may happen in the mammalian cell culture laboratory. So, various safety instruments such as fire extinguishers, fire blankets, emergency showers, etc. and their usefulness are discussed in this chapter.

Understanding the various materials needed to set up a mammalian cell culture laboratory is the primary focus of this chapter. Additionally, requisition for safe and contamination-free handling of mammalian cell culture and understating maintenance of the safe environment both for the laboratory personnel and the laboratory itself is the basic theme of this chapter.

2 Basic Requirements of a Cell Culture Laboratory

The prerequisite of mammalian cell culture is a well-lit and suitably ventilated laboratory with the highest level of sterility.

Another important requirement of a cell culture laboratory is the various instruments necessary for the isolation, culture, and maintenance of mammalian cells.

Before working in a mammalian cell culture laboratory, the following points must be remembered:

- The cell culture room should be restricted with limited people from outside to avoid microbial contamination in cultures which is normally a major problem of cell culture.
- A room shall be entirely dedicated only for performing mammalian cell culture.
- The mammalian cell culture laboratory must be kept clean and dust-free using sealed windows, fitted with high-efficiency particulate air (**HEPA**) filter.

  NB: HEPA is an acronym for high-efficiency particulate air or high-efficiency particulate arrestance. This acronym refers to a filter that is manufactured, tested, certified, and labeled by current HEPA filter standards.
- No yeast or bacterial or other microbial culture activities should be allowed in the mammalian cell culture laboratory.
- All the biohazard wastes should be disposed of regularly in a proper manner and taken away outside the culture room for proper destruction using the sterilization unit.
- The more sterile activities are kept away toward the back of the laboratory using an anti-room between the cell culture room and the main entrance.
- There should be the provision of either fumigation or heat decontamination of the whole laboratory if and when it is required to remove any contamination.
- Any cell culture laboratory should be equipped with a **laminar flow hood, CO_2 incubator, water bath, bench top centrifuges, and** temperature-controlled **centrifuges**.
- A cell culture laboratory should also have a **hemocytometer, an automated cell** counter, and a regular and **inverted microscope** fitted with a camera and computer.
- There must be provisions for mammalian cell culture-grade culture vessels (made up of polystyrene) and accessories, sterile glass and plastic wares, biohazard waste containers, and disposal.
- **Cell culture facility should be always adjoined with an outside regular laboratory** housing the sterilizing oven and autoclave; water purification system; refrigerators; −20 °C and −80 °C deep freezers for keeping reagents, culture medium, cells, etc.; and liquid nitrogen containers for keeping live cells in a frozen state for an indefinite amount of time (Mather and Roberts 1998; Morris 1998; O'Connor and O'Driscoll 2006; Wesselschmidtm and Schwartz 2011).

  NB: All the instruments necessary for the mammalian cell culture laboratory are discussed in chapter ▶ "Mammalian Cell Culture Laboratory: Equipment and Other Materials."

3 Setting Up of a Cell Culture Laboratory

While building out a new laboratory space, the design team usually includes an architect, a contractor, a builder, an electrician, a mechanic, a plumber, and a laboratory director or manager. Certain rules must be followed for the designing of a mammalian cell culture laboratory, such as the following:

- All cell culture laboratories must contain several basic facilities. **Cleanliness is the major criterion for fundamental design apart from the routine maintenance of different cell culture facilities**.
- Thus, the most urgent concern in developing a mammalian cell culture laboratory is the proper usage of biological safety cabinets and rigorous antiseptic techniques.
- For contamination-free culture, the main cell culture room **should be designed in such a way that it would be entirely dust-free, clean, and environmentally controlled**.
- **Windows should be properly sealed to** keep the inside environment dust-free. **Rust-free, swinging aluminum double doors should be arranged in entire passage ways one after another that could be opened hands free either by the push of the body or via feet**.
- **Laboratory materials including walls of the rooms or even chairs, tables, etc. should be made up of such materials that routine cleaning of the cell culture facility with disinfectants and aseptic procedures such as 70% ethanol** can reduce the contamination to less than 1%. All cell culture laboratories should have easily washable walls and floors.
- Ideally, there should be **an anti-room in between the cell culture room and the main entrance**, to keep away any outside dust particles that can enter the main cell culture facility.
- There should be a provision of **central air conditioning** to lower the temperature for a cool and comfortable working environment.
- Fine-wired electrical lines, telephone lines, fire alarms, and an emergency generator should be installed in the cell culture laboratory for safety and security.
- It is best to design a cell culture laboratory that is not accessible directly from the outside of a building.
- The medium preparation area, glassware washing area, or storage area should be l**ocated outside the main cell culture room**.
- **Ideally, the medium preparation and sterilization area lead inside toward the anti-room crossing the aseptic transfer area, eventually leading to the innermost cleanest cell culture room**.
- The length and total area of a cell culture laboratory depend on the biosafety level, the number of people who will work in the laboratory, the number of instruments that need to be accommodated, and the total budget allocated to set up a mammalian cell culture laboratory (Morris 1998).

In addition to the main cell culture facility and anti-room, the outer laboratory should be divided into the following areas for routine purposes (Fig. 1):

1. A general washing area
2. A sterilization, media preparation, and storage area
3. An anti-room
4. An aseptic transfer area and main cell culture room
5. An observation/data collection area

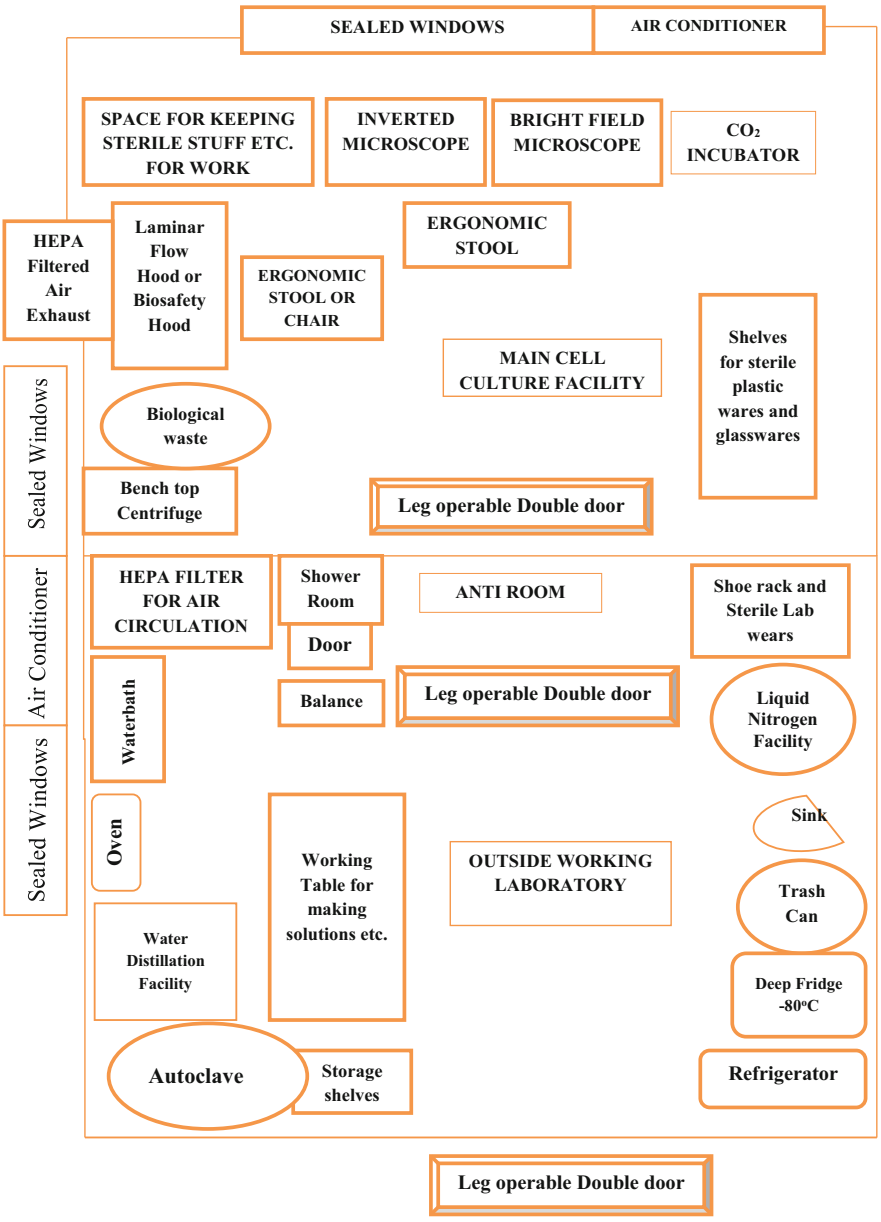

Fig. 1 Block diagram depicting the constitution of a mammalian cell culture laboratory

The following describe the various areas of a mammalian cell culture laboratory.

3.1 General Washing Area

- The general and glassware washing area should be located next to the sterilization and storage area so that the glassware can be readily sterilized and stored after washing. **Acid baths, automated dishwashers (optional), pipette washers and driers, drying ovens or racks, and storage cabinets** should be kept here.
- After work, all glassware and plasticware used for culturing are removed and are often autoclaved in the adjacent sterilization area to destroy the cell residues and contaminants and to soften the semi-solid medium. Thereafter, they are taken to the sink area for further washing and cleaning.
- The washing area should be fitted with **large sinks** and draining boards, all lead-lined with acids and alkali-resistant PVC. Both **hot and cold water** should be available for washing in the sink.
- Single distilled water, double-distilled water, and **cell culture-grade deionized (Millipore) water** should be readily available for use.
- All containers should be rinsed with single- and double-distilled water and cell culture reagents must be made with cell culture-grade deionized (Millipore) water.
- **All glassware and plasticware (autoclavable) used in cell culture must be immersed in chromic or hydrochloric acid water in acid-resistant plastic tubs overnight**. Thereafter, this glassware and plasticware should be thoroughly washed many times in running tap water to remove even a bit (trace) of acid before being rinsed with single- and double-distilled water respectively.
- **The glassware and plasticware used for cell culture facilities should never be washed with detergent as this can hamper the attachment and growth of the cells.**
- After washing, all the glassware should be **dried in a hot oven**.
- The plasticware should be dried **in open air** or **37 °C dry incubator**.

3.2 A Sterilization, Medium Preparation, and Storage Area (Table 1)

- Next to the glassware washing area, the sterilization, medium preparation, and storage area should be located.
- The storage area should have ample storage space and cabinets for keeping the chemicals, culture vessels, sterile plasticware, and glassware required for medium preparation and dispensing.
- An 8-feet-high room generally accommodates five shelves, each 18 inches apart, where the bottom shelf is 4 inches off the floor.

Table 1 Various small instruments necessary for sterilization, medium preparation, and storage area

Name of equipment	Equipment specification	Specific function
Water purification system	Should have a resistivity of at least 200,000 ohms-cm and a conductivity of 5.0 micromhos cm^{-1}	Purification of deionized water for medium preparation
Electronic rough balance	0.1–200 g maximum capacity	Measuring out reagents and medium materials
Electronic microbalance	2–20 m minimum capacity	Measuring out fine reagents and medium materials
pH meter	pH range, 0–14 +/− 0.01; automatic temperature compensation, 0–600 °C (one- or two-point calibration)	Measurement and adjustment of medium pH
Magnetic stirrer with hotplate	Variable heating range from ambient to 400 °C; variable stirring speed from 50 to 150 rpm; chemically resistant	Mixing and heating medium and stock
Refrigerators	Temperature: 0–5° C	Storage of stock solutions, medium, etc.
Freezers	Temperature: 0 to −20 °C	Storage of enzymes, proteins, hormones, etc.
Ultra-deep freezer Liquid nitrogen containers	Temperature −80 to −150 °C −196 °C	Storage of RNA, growth factor, cells, etc. For permanent storage of cells
Autoclave	The temperature of **121 °C and 15 pounds per square inch (psi) for 15 min**	Moist heat sterilization is performed at a high temperature as the **spores of fungi and bacteria** can be killed at **high pressure and temperature**
Temperature-controlled shaker water bath	Temperature maintained from 10 °C to 100°C	Thawing of frozen mediums, quick thawing of frozen cells, de-complementation of serum, etc.

- The storage cabinets should be dust-proof and lowly located for easy access. Light material like wood or plywood storage should be preferably employed in making cabinets. Expanded metal storage cabinets with 1/4 or 1/2 inch wire mesh providing **better air circulation** can be also used as an alternative.
- A variety of basic laboratory instruments should be available in the medium preparation area, such as *a good temperature-controlled refrigerator and freezers for storing stock solutions and chemicals, a rough balance that measures up to 200 g, a fine microbalance that measures up to 2 mg, hotplate* cum *magnetic stirrer, pH meter, water baths, air vacuum sources, Bunsen burners fitted with a gas source, a microwave, or a convection oven*.
- A**n autoclave or domestic pressure cooker** for **moist heat** sterilizing medium, glassware, and instruments is also needed.

NB: Generally mammalian cell culture media contains temperature-sensitive materials such as amino acids, growth factors, etc.*; therefore, instead of autoclaving, filter sterilization is preferred.*

- This area should have ample provisions for storing single- or double-distilled water as well as deionized Millipore water. **Water used for making all cell culture reagents and medium should meet a minimum standard for type II reagent-grade water, i.e., free of pyrogens, gases, and organic matter (cations/anions)**. **The type II grade water should have an electrical conductivity of less than 1.0 μmho cm^{-1}**.

  NB: If space is available, a separate room may be dedicated for liquid nitrogen containers.
- *An alarm system and backup generator may also be very useful if the budget permits.*
- *The interior of the cell culture facility may have the following finishing: vinyl flooring, nonporous ceilings, washable impermeable paints, coatings, benchtops, and furniture*.

3.3 Anti-room

- An anti-room in front of the actual cell culture room should be always designed to limit outside to inside airflow as it may contain microbes and spores.
- This room should be as clean as possible.
- *All cell culture workers should leave their shoes outside this anti-room to keep dust at a minimum.*
- *Special (either disposable or autoclaved sterile) laboratory gloves, shoes, and coats should be worn in this area.*

3.4 Aseptic Transfer Area and Main Cell Culture Facility

The main cell culture facility should be regulated to ensure the highest possible cleanliness, according to the workflow and traffic pattern.

The aseptic transfer area and the main cell culture laboratory are characterized by the following optimizations.

3.4.1 Temperature, Relative Humidity, and Light

- In the cell culture facility area, all windows should be sealed as open windows pose contamination problems in the summer and humidity problems in the winter.
- So, to keep comfortable room temperature around 76 °F inside the culture facility, the use of room heaters, heat pumps in winter, and air conditioners or exhaust fans in summer is preferred. Several electric outlets need to be installed for accommodating **laminar flow hoods, CO_2 incubators, microscopes, and occasionally a** flow-activated **cell sorter (FACS)**.

- All cell culture facilities should be equipped with abundant artificial lights with well-regulated electrical wire connections.

3.4.2 Surfaces, Doors, Windows, and Storage Spaces

- All surfaces inside the cell culture facility should be smooth and clean.
- Ideally, all cell culture techniques can be performed on an open laboratory bench if the air is **dust- and** microbe-free, **absolutely clean, and dry.** However, in reality**, this type of air quality is not practically available for work.** Therefore, the surfaces, walls, and doors should be designed and constructed in a manner that dust and microorganisms do not settle and can be easily wiped clean with suitable disinfectants for regular purposes.
- All windows should be sealed. Doors should be operable hands-free and should always be properly closed.
- Ample shelf spaces are organized to store sterilized materials and ready-to-use containers.

3.4.3 Laminar Flow Hood (Fig. 2)

- The simplest and smallest transfer area suitable for cell culture work is an enclosed plastic box fitted with a UV light, normally referred to as a **glove box**. A **laminar flow hood** with the **provision of filtered sterile air or a sterile complete transfer room is** used for routine aseptic cell culture work. However, for higher sterility and good laboratory practice (**GLP**), a good quality laminar flow hood is installed inside the sterile cell culture room.
- To ensure a dust-free clean environment, this type of sterile room and laminar flow hood should be fitted with **an overhead ultraviolet (UV) light for disinfection and a positive-pressure ventilation unit attached with a high-efficiency particulate air (HEPA) filter**. UV lights are used for disinfection only when people and live cells remain outside the facility. During entrance from outside, the UV lights are first shut off and regular lights are turned on by installed safety switches.

Fig. 2 Front (**a**) and side (**b**) views of laminar air flow (LAF) hood

- A 0.3 μm HEPA filter works well with a 99.97–99.99% efficiency. Before passing through a HEPA filter, normal air is first forced through a dust filter. The air is then filtered either downward (**vertical flow unit**) or outward toward the front (**horizontal flow unit**) over the working surface by a HEPA filter. The constant flow of clean, filtered air pushes out the unfiltered air and all particles away from the work surface.
- All working surface areas should be thoroughly disinfected with **70% ethyl alcohol (ethanol)** before and after work.
- The laminar flow hood and sterile cell culture room should be equipped with a source of **electricity, gas, compressed air, aspirators,** and a **vacuum**. Aspirators and vacuum pumps are used for **filter sterilization** of culture media and solutions.
- Pipettor and multichannel pipettor are necessary for handing adequate volume of cells with culture medium (in suspension) (Bykowski and Stevenson 2008).

3.4.4 Ergonomic Design of the Laminar Flow Hood and Sitting Chair

In a cell culture facility, biosafety cabinets are used for long hours daily to provide primary protection to both workers and the cell culture products. Workers in cell culture facilities are often exposed to **unhealthy circulations**, due to elongated bad sitting posture and boredom of working in a closed environment. Therefore, the ergonomic **design of the biosafety cabinet** is highly important for ensuring the comfort and convenience of the workers.

The following parameters are highly recommended to improve working conditions as well as the smooth functioning of a cell culture laboratory:

- According to standard ergonomic design, the work surface top, as well as knee space, should be at least 30″ deep, 60″ wide, and 19″ deep by 30″ wide by 27″ high, respectively.
- According to the standard comfortable work rule, the distance a researcher must work should be the natural 8–14″ range. Based on this rule, the researchers must position themselves very close to or in proximity to the working table so that there are no obstructions in the working environment and the work conduct must be as comfortable as it could be.
- Air conditioning and heating system (for maintaining comfortable ambient temperature).
- Reduction of overall noise level (for a peaceful working atmosphere).
- Panoramic side windows to provide more natural light.
- The light source should be such that the working surface would be suitably illuminated, without any obstruction by any objects. An ergonomic adjustable footrest that allows for comfortable positioning beneath the cabinet could improve posture and comfort.
- Armrest. Using an armrest across the cabinet front improves the support and comfort.

An ergonomic workstation requires certain criteria to select a desired working chair with the following characteristics:

- A five-leg chair is recommended, as and when the chain is in casters.
- Following sitting, castors must move easily and comfortably without any hindrance as and when the movement is required.
- While sitting, seat height must be adjustable, if required.
- An easy-to-swivel seat is a requirement.
- Adjustable (**0–10**°) slanted seat is needed for a comfortable working rule.
- The front edge should be padded and contoured into a waterfall design behind the knees.
- Finally, the seat width should be sufficient enough (17–20″) to permit the back of the working person to contact the lumbar backrest without impinging nerves in the back of the knees.

3.5 Observation and Data Collection Area

The observation and data collection room should be adjacent to the main cell culture room that contains the following materials:

- A CO_2 incubator.
- A bright-field microscope or an inverted microscope equipped with a camera and computer.
- Some laboratories may need a phase contrast and a dissecting microscope or even fluorescent and confocal microscopes.
- A flow cytometer for cancer biologists/immunologists/cell biologists.
- A computer and writing desk to note the research data on growing, viable cells (Mather and Roberts 1998; Morris 1998; O'Connor and O'Driscoll 2006; Wesselschmidtm and Schwartz 2011).

4 CO_2 Incubator and Environmental Considerations

- Every mammalian cell needs an optimum environment for its culture and growth in the cell culture laboratory. In general, mammalian cells need an environment (medium) where all the six components of nutrients (**carbohydrates, proteins, fats, vitamins, minerals, and water**), pH of 7.4, a temperature of 37 °C, moisture of 95%, adequate osmotic pressure, O_2, and CO_2 tension are maintained.
- The cell culture laboratory and the cell culture materials (e.g., medium) should be designed in such a way that the **physicochemical environment (temperature, pH, osmotic pressure, and O_2 and CO_2 tension) and the physiological environment (growth factor, hormones, amino acids, glucose, etc.)** of the cultured cells must be maintained. There are automated, programmable CO_2 incubators available that can be used as and when there is a need for decontamination; a simple switch to turn on the program is required (Tamoaki et al. 2002; Kempner and Felder 2002).

The following three major factors affect the mammalian cell culture growth environment:

1. Medium for cell culture
2. CO_2 levels and pH
3. Temperature

Here is a brief discussion of the above three factors.

4.1 Medium for Cell Culture

- For in vitro culture of mammalian cells, the culture medium is highly essential. It not only supplies all the necessary nutrients (including growth factors, hormones, and amino acids) but also maintains optimum CO_2 (5%), humidity (95%), pH (−7.4 to 7.5), and osmotic pressure (280–330 mOsmol kg^{-1}) extents needed for the cell growth.
- Both natural and defined media are used for cell culture. While the natural medium is designed based on tissue extracts and various body fluids, the defined medium is artificially synthesized based on the specific requirement of the cultured cells.
- In the above context, three basic classes of media are **basal media**, **reduced-serum media,** and **serum-free media**, respectively. These media differ in their requirement for serum supplementation. A detailed discussion of the culture medium is made in chapter ► "Common Reagents and Medium for Mammalian Cell Culture" (Eisenblatter et al. 2002; Freshney 2010; Biocompare 2019).

4.2 CO_2 Levels and pH

- While mammalian cell cytoplasm maintains a pH of 7.2 with a reduced redox environment, the blood pH is 7.4.
- Since blood plasma supplies all the nutrients to the human cells, the pH of the plasma should be taken as a standard for experimental cell culture research. Thus, the in vitro cell culture conditions a standard pH of 7.4.
- In contrast, while some normal fibroblast cell lines prefer a slightly basic culture medium (pH 7.4–7.7), some transformed cell lines prefer a slightly acidic environment (pH 7.0–7.4).
- During the normal metabolism of the cells, several toxic metabolites (e.g., lactic acid) may accumulate in the culture medium, which may alter the pH of the cell culture medium.
- Since unnecessary alteration of cell culture medium pH may drastically alter the cultured cell's capacity to proliferate as well as propagate normal physiology, maintaining the buffering capacity is highly essential.

- In this connection, the vast majority of the mammalian cells use 5% CO_2 (a few may use 4–10%). Upon entering the cell culture medium, CO_2 reacts with medium water and is converted to H_2CO_3 (carbonic acid). Since H_2CO_3 is a weak acid, it spontaneously breaks down to $H^+ + HCO_3^-$ and therefore maintains the pH of the culture medium.
- Additionally, to increase the buffering capacity of the cell culture medium, bicarbonate or HEPES buffer may be used.

4.3 Temperature

- In physiological conditions, the center of the peripheral nervous system, i.e., the hypothalamus, maintains a temperature of 37 °C. So, technically, 37 °C is the ideal temperature for in vitro culture of mammalian cells in a CO_2 incubator.
- In general, there may be rarely a trivial alteration (1–2°) in the temperature, but most of the standard CO_2 incubators maintain the said temperature of 37 °C. It is a well-known fact that over-heating may have a more serious effect on the cultured cells than heating. Therefore, in general, the CO_2 incubator temperature is set at 36 °C.
- Besides maintaining the cell culture environment, it is necessary to maintain the proper working environment of the cell culture laboratory.

In this aspect, three major factors are discussed:

1. Location
2. Gases
3. Ventilation

Here is a brief discussion of the above factors:

4.3.1 Location

The mammalian cell culture laboratory must be maintained **clean, free from dust, and in an easy-to-disinfect area**. To prevent any type of contamination, the laboratory area must be restricted to the cell culture personnel only.

4.3.2 Gases

In general, mammalian cells are cultured at 5% CO_2. However, some mammalian cells require between 3% and 10% CO_2. It is generally recommended that instead of one CO_2 cylinder, two cylinders are connected with the CO_2 incubator. While one cylinder is used for the main line of gas supply, the other is used for backup works. This prevents any sudden change of CO_2 pressure as and when one cylinder is about to get exhausted. Additionally, with two cylinders in place, the incubator can be left for weeks or even months (depending on the use) without any replacement with new filled cylinders. Cylinders must be fixed with a solid support or wall in such a way that there should not be any accidental fallout. For this purpose, automatic

changeover units can be incorporated into the external CO_2 supplies. It is always better not to have large cylinders of gas in a culture room for safety, practical, and aesthetic reasons (Angerman 1999; Farré et al. 2018).

NB: One of the important mild buffers used in the cell culture is 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (**HEPES).** It has been observed that some carefully controlled incubators use HEPES buffer to maintain the cell culture pH, without any additional CO_2 supply. However, for a large number of mammalian cell cultures, a CO_2 supply is a must to control the pH of the cell culture medium.

4.3.3 Ventilation

The ergonomic and aesthetic design of a cell culture laboratory with specified airflow is necessary to circulate clean and dust- and dirt-free air through a **HEPA filter**. For this purpose, windows must be sealed to prevent the entry of external air with dirt, dust, and environmental germs. Thus, all the air must pass through the HEPA filter only.

There must be an option for fumigation either for the whole room or just for a single cabinet. Fumigation must be operated before changing the HEPA filter. This will eventually make the cell culture room germ-free. There must be an option to eliminate the inside air to the outside of the cell culture medium, without contaminating any laboratory personnel. It is claimed that HEPA filtration is a statistical process (99.999–99.97% efficiency, depending on the manufacturer).

- According to CDC rule 1999, in larger cell culture facilities, careful balancing of air pressures in culture and anterooms might be useful. In this case, both negative and positive air pressure must be balanced in a proper way (CDC 1999).
- Balancing and filtration require careful installation and validation at the outset besides continued regular maintenance and monitoring to ensure appropriate function. Ventilation systems will also need to be tied into building fire management systems, for example, automatic smoke dampeners to prevent the ventilation system from fanning or spreading smoke and fire.
- *NB: Liquid nitrogen is used for the permanent storage of mammalian cells. The cells can be preserved both in the vapor and liquid phases. Accidental spillage of liquid nitrogen may cause severe injury to humans, besides a likely reduction in the oxygen level in the air. Therefore, cell culture room ventilation should be adequate to support instant evaporation of liquid nitrogen whereby oxygen concentration in the cell culture room should be maintained at least 14%. If this cannot be guaranteed at all times, oxygen monitoring and alarm equipment are indeed needed* (CDC 1999; Tamaoki et al. 2002a, b; Kempner and Felder 2002).

4.4 Principle of Risk Assessment and Types of Risk to Mammalian Cell Lines and Their Handling

A cell culture laboratory has many **specific handling hazards besides the regular risk of electrical and fire hazards**. Handling hazards are **of two types: biohazard or handling of different human or animal cells and tissues and infectious organisms and chemical hazards, i.e., handling of toxic, corrosive, or mutagenic solvents and chemical reagents**. Additional handling hazards include inhalation exposures to infectious aerosols, ingestion through mouth pipetting, accidental punctures with syringe needles or other contaminated sharps, and spills and splashes of cells or chemicals onto the skin and other mucus membranes (Frommer et al. 1993).

Thus, safety features in a cell culture laboratory typically are of prime importance and should be ideally in strict adherence to standard mammalian cell culture practices and techniques. Figure 3 depicts the international biohazard warning signal. The following sections describe the basic principle of risk assessment and the types of risk associated with the mammalian cell lines.

4.4.1 Principles of Risk Assessment

The four fundamental principles of risk assessment are as follows:

1. Prevention of injury to workers.
2. Ensure that individuals are not subjected to hazards.
3. Protection of the laboratory properties.
4. Avoidance of long-term harm to individuals and the environment.

4.4.2 Types of Risk

While culturing mammalian cells, certain risks associated with health and physiology can present to the laboratory personnel.

Based on the level of risk, these are divided into three following classes:

Fig. 3 International biohazard warning signal

Low risk: Well-characterized cell lines which are already examined for risk assessment for human health and physiology are called low-risk cell lines. In this classification, a large number of nonhuman or nonprimate cell lines are included.

Medium risk: In this class, all the poorly classified cell lines are included.

High risk: Highest precaution should be taken to prevent human health injury and/or contamination while working with these cell types. The cells included in this category are primary cultured mammalian cells including those derived from mammalian blood and cell lines that are created using the pathogenic viruses and cell lines that may be infected with various pathogens. To properly handle these cell lines, the Advisory Committee on Dangerous Pathogens (**ACDP**) guidelines must be strictly followed. The university guidelines may vary a bit as stipulated by the governments of various countries.

4.5 Concept of Biosafety and Biosafety Levels

- A biosafety hood or cabinet is defined as **an enclosed, ventilated workstation for safe working with biologically hazardous materials or potentially harmful microbial pathogens requiring designation by a biosafety level**. Several biosafety cabinets exist depending on the biosafety levels, as mentioned in the below sections.

4.5.1 Basic Difference Between a Laminar Flow Hood and a Biosafety Hood

- In a laminar flow hood, unfiltered exhaust air blows out to the environment causing risk to the laboratory personnel and the environment itself, whereas in a biosafety hood, all the exhaust air is HEPA filtered to remove harmful bacteria, yeast, and viruses so that the released air is safe for the environment.
- **However, due to the absence of HEPA-filtered air in a fume hood, a normal chemical fume hood cannot be used as a biosafety hood and vice versa**.
- The safety measures mainly include **following strict guidelines in day-to-day work to protect from harmful accidents in cell culture laboratory settings and regular monitoring of biosafety reviews in the laboratory**.
- **For high-security facilities, biosafety practices are learned, followed, and extended beyond the cell culture facility** (Richmond and McKinney 1999).

4.5.2 History of Invention of Biosafety Level

The most important years in the history of the invention of biosafety cabinets are 1943, 1995, 1957, 1966–1976, 1984, 2006, and 2015.

Here is a brief description of the importance of the above years:

- **1943:** The history of biosafety level invention dates back to the prototype **Class III biosafety cabinet** in 1943 by US Army soldier ***Hubert Kaempf Jr.*** under the directions of *Dr.* ***Arnold G. Wedum***, Director (1944–1969), Industrial Health and Safety at the US Army Biological Warfare Laboratories, Fort Detrick, Maryland.

This was reportedly the first maximum biosafety level containment mentioned, until the meeting of 14 representatives on 18 April 1955, at Fort Detrick in Frederick, Maryland.

- **1955:** In this meeting held in Maryland, knowledge and experiences regarding biosafety and chemical, radiological, and industrial safety issues were exchanged, which was common to the operations at three principal US Army biological warfare (BW) laboratories. These conferences discussed the top-level security clearances owing to concurrent potential biological warfare threats.
- **1957:** In 1957, these conferences opened the door to broader sciences for sharing biological safety information in the context of potential threats. Over the next decade, the representatives were invited from all federal agencies for sponsoring or conducting research on **pathogenic microorganisms**.
- **1966–1976:** Scientists, professors, and researchers from universities, private laboratories, hospitals, and industrial complexes were invited, and in 1983, a formal organizational setup was discussed.
- **1984:** The ***American Biological Safety Association*** (**ABSA**) was officially established alongside the drafting of the biosafety constitution and bylaws.
- In the United States, the regulations and recommendations for biosafety levels were first time mentioned in the document "**Biosafety in Microbiological and Biomedical Laboratories,**" prepared by the **Centers for Disease Control and Prevention (CDC)** and the **National Institutes of Health (NIH)**, published by the **US Department of Health and Human Services**. The document describes four ascending containment levels, referred to as biosafety levels, ranging from 1 to 4, depending on the risk of microbiological practices, use of safety equipment, and risk-level safeguard facilities associated with biological material handling. The European Union (EU) also has a similar biosafety concept, exercisable from levels 1 to 4.
- **2006:** As of 2006, there are four safety levels. These are noted as **BSL-1 to BSL-4 in the United States** or **P1 to P4 (for pathogen or protection level) in Europe**.
- **2015:** As of 2015, **ABSA** comprised more than 1600 professional members from different parts of the world.

4.5.3 Use of Biosafety in the Modern Scientific Laboratories

- **The primary purpose of a biosafety cabinet is dual**. The first of these rationales is obviously to protect the laboratory personnel and the surrounding environment from microbial pathogens. The second major function is the feasibility of work continuation without yeast, bacterial, viral, or any other contamination from the surrounding environment. Therefore, the HEPA-filtered air in the biosafety hood ensures the highest sterility of mammalian cultures (Cote 2001).
- All cell culture work is performed carefully and safely in a biosafety cabinet. CDC recommends best laboratory practices to reduce and control spills, splatter, and aerosol generation to minimize the personnel exposure and contamination risk during cell culture.

- The laboratory members are advised to keep clean materials at least 12 inches (**30 cm**) away from aerosol-generating sources and arrange the work flow inwards **from clean to contaminated**.
- *Open flame is not necessary within the clean environment of a Class II or III biological safety cabinet as it may disrupt the airflow.*
- Upon completion of work inside a biological safety cabinet, its surface along with other laboratory equipment and materials is periodically decontaminated and disinfected.
- The usefulness of biosafety is not limited to just mammalian cell culture laboratories and, perhaps, covers any laboratory where live pathogenic creatures or even viruses are cultured.
- Besides the CDC and NIH in the United States, various European organizations have set up rules and regulations for the safety of the laboratory personnel in a mammalian cell culture laboratory (Human Tissue [Scotland] Act 2006).

4.5.4 The Use of Biosafety May Be Immensely Helpful in the Following Areas of Present Society

- In ***ecology,*** while handling imported life forms from beyond ecological region borders.
- In ***biotechnology and genomics,*** reducing the risk of alien viral or transgenic genes or prions.
- In ***food technology***, for combating the risk of bacterial contamination.
- In ***medical research especially in virology and immunology***, while handling normal or infected organs or tissues or genetic therapy products, infectious viruses, bacteria, protozoa, etc. Biosafety levels of laboratory containment protocols are subcategorized as 1–4 in the order of rising hazard and associated danger (West et al. 1978).
- In ***biochemistry***, i.e., carcinogenic nitrates, arsenic levels in the water, or toxic chemical agents like SDS (an anionic surfactant).
- In ***space biology*** due to NASA's policy for handling space samples containing alien microbes in **special higher BSL-5 conditions.**
- In ***synthetic biology*** (the next-generation tool of modern biology), synthetic vaccines are now being developed on a large scale, in less time, and at a lower cost. These synthetic vaccines and antibodies can also enhance virology and immunology knowledge (Herman and Pauwels 2015).

  NB: It is estimated that in near future, man-made unicellular organisms could be produced in the Biotech laboratory for creating biofuels and destroying harmful atmospheric pollutants.
- However, it is also anticipated that these newly synthesized organisms will have a significant effect on prevailing biomass. These synthetic organisms can harm existing life by disturbing the food chain(s) or reproduction between species as well as competing against other species that are at risk via acting as invasive species.
- Scientists promoting **synthetic biology** argue in favor of synthetic organisms so that the use of biosafety mechanisms such as **suicide genes and nutrient**

dependencies could ensure their nonsurvival outside the lab environment where these were created.

- Therefore, strict biosafety containment levels should be emphasized to control the environmental release of these organisms.
- Organizations like the **ETC Group** argue that biosafety regulations should aim at the creation of organism(s) that could potentially harm existing life.
- **The international Cartagena Protocol on Biosafety** not only primarily deals with agricultural biosafety but also extends to postgenetic threats involving artificial life forms, new molecules, and even robots that may compete directly in the natural food chain.
- During biological warfare, involving new biological threats (i.e., **robots, new artificial bacteria, or viruses**), **biosafety** precautions are not sufficient.
- So, new terminology of **biosecurity** is introduced to arrest the bioterrorism threat.

4.5.5 Biosafety Levels

Typically, institutions that experiment with or create potentially harmful biological material(s) do have a committee or board of supervisors, as per the clause of the institution's biosafety measures. **The Institutional Biosafety Committee** drafts guidelines to monitor the laboratory biosafety standard to prevent the accidental exposure of potentially dangerous biological agents. **Biosafety level (BSL)** is referred to by the **strict adherence to different biosafety precaution levels (BSL-1 to BSL-4),** required to **isolate potentially dangerous biological agents and biohazards** (Barkley 1979).

Biosafety level 1
Biosafety level 2
Biosafety level 3
Biosafety level 4

NB: Besides the above four BSLs, NASA developed special biosafety called "biosafety level 5" for handling space samples containing alien microorganism species.

4.5.6 Rationale of Biosafety Numbering

- Biocontainment can be classified as the relative threat to the safety of the surrounding environment as BSLs, ranging from level 1 to 4.
- **Higher biosafety levels infer a greater risk from the external environment including everyone working in the laboratory**.
- At the lowest BSL, the containment zone may only comprise the simplest laminar flow hood or chemical fume hood.
- The highest BSL involves the isolation of an organism from the surrounding environment via isolated buildings, sealed rooms, sealed containers, positive pressure personnel suits (sometimes referred to as "spacesuits"), and elaborate procedures for entering the room beside the cautionary measures before leaving the rooms. This includes the highest security levels for accessing the facility,

restricted to the admission of authorized personnel. Therefore, this is considered **restricted access and a hot zone**.

- **The hot zone must be preserved and maintained with extreme cautions so that there should not be any incidental chance of dangerous pathogens getting leaked into the external environment** (CDC Biosafety Cabinets 1995, 1999, 2000, 2008, 2009).

Here is a brief description of the above four biosafety levels 1–4.

Biosafety Level 1

Biosafety level 1 is characterized in the following lines:

- The Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, nominated the biosafety level 1 (**BSL-1**) in 1997 as the minimal protection norms for common basic research and clinical laboratories posing minimal potential hazards to laboratory workers and the external environment.
- Thus, the BSL-1 level is the threshold basic safety standard needed for minimizing the threats caused by nonpathogenic **biological, physical,** and **chemical agents in normal, healthy human adults.**
- So, the BSL-1 laboratory is **not necessarily separated from general workers in the main laboratory.**
- The work can be routinely carried out in a corner of the main laboratory on a dedicated open bench space or inside a basic laminar flow hood.
- Workers in the BSL-1 laboratory should follow **common biological procedures** while working in the laboratory.
- All workers should use **disposable hand gloves** and **face masks** while handling chemicals and biological agents. Additionally, there should be adequate safety arrangements for accidental spills and exposure.
- In BSL-1, several nonpathogenic bacteria such as *Escherichia coli*, *Gram-negative bacteria* (some strains of *E. coli* may cause stomach infection), and nonpathogenic viruses, and some **innocuous cell cultures** can be safely handled without any severe harm.
- Decontamination procedures for BSL-1 include basic cautions against daily life microorganisms like washing hands with antibacterial soap and wiping work surfaces with disinfectant including 70% alcohol, before and after work.
- Contaminated materials are routinely destroyed in an autoclave before being disposed of in open. The discard containers must be separately indicated and properly marked so that the disposal end courses are optimally distinguished.
- These biosafety cabinets are either un-dusted either via connecting with the building exhaust system or recirculating exhaust air back into the cell culture laboratory.

  NB: BSL-1 provides safety to laboratory personnel and the environment but no protection to microbial culture(s). Practically, the inward airflow (minimum velocity of 75 feet/min) can cause sample contamination.

Biosafety Level 2

- Biosafety level 2 **(BSL-2)** is almost similar to BSL-1, except for working with agents that are **moderately hazardous to laboratory workers and the environment**.
- Therefore, a BSL-2 laboratory requires **isolation from the main laboratory** with a provision of anti-room and restricted access to experienced workers, well trained in handling pathogenic agents.
- *Most cell culture laboratories should be at least BSL-2,* although exact safety requirements may vary concerning a specific cell line usage and the research type.
- BSL-2 work involves handling various pathogenic bacteria and viruses that either cause only mild diseases in humans or **are difficult to contract via aerosol in a laboratory setting,** such as *Clostridium difficile*, mostly *Chlamydiae*; *Hepatitis A*, *B,* and *C* viruses; *Orthopox* viruses (other than small pox); mumps and measles viruses; etc. All BSL-2 bacteria and viruses are moderate-risk agents capable of initiating mild to severe diseases either by direct ingestion or through mucocutaneous membrane exposure.
- In a BSL-2 laboratory, infectious aerosols are created during the aseptic procedure. Therefore, work should be performed carefully in BSL-2 cabinets.

Suggested Precautions When Working with Infectious Agents in BSL-2 (Fig. 4)

- **Extreme precautions** should be taken while handling and disposing sharp items contaminated with BSL-2 bacteria and viruses.
- The use of mouth or hands should be completely avoided when transferring infectious materials.
- Electrical or manual auto-pipettes should be used for transferring such materials and should be disposed of properly after use.

NB: BSL-2 cabinets are commonly used in research and clinical laboratories.

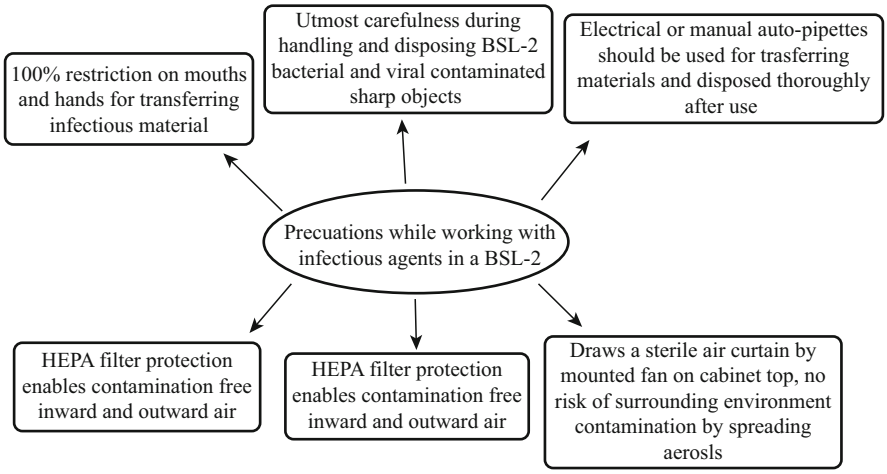

Fig. 4 Summary of BSL-2 precautions during laboratory working with infectious agents

- BSL-2 provides safety to both the mammalian culture and environment since both inward and outward air are HEPA filtered.
- In principle, all BSL-2 cabinets operate by drawing a curtain of sterile air through a fan mounted on the cabinet top, over the work surface, and back up to the top of the cabinet, where it passes across the HEPA filters. Thus, the mammalian cell cultures on the work surface remain completely sterile and cannot contaminate the surrounding environment by spreading aerosols.
- In a BSL-2, the exhausted air is compensated by air being drawn into the cabinet front, from below the work surface. Together with the sterile air curtain, the redrawn air acts as a potential safety barrier forbidding the contaminated air from reaching back out to the workers.

Biosafety Level 3 (Fig. 5)

- Biosafety level 3 (**BSL-3**) is used for handling the potentially more dangerous biological agents that can cause **more serious and potentially lethal infections** even via **aerosol inhalation or epidermal exposure**.
- Laboratory personnel must have a clear understanding of the type of pathogens kept at bay using the BSL-3 conditions. In general, highly infectious, disease-causing pathogens that are previously implicated in a large-scale pandemic, leading to a significant number of human deaths, are included in this category. This list includes the recent pandemic causing SARS-CoV-2, multidrug-resistant *Mycobacterium tuberculosis*, *Yersinia pestis* (causative agent of plague), yellow fever, Brucella, rabies, West Nile viruses, chikungunya, and so on.
- The main research building/other buildings must be separated from the BSL-3 laboratory through an anti-room.

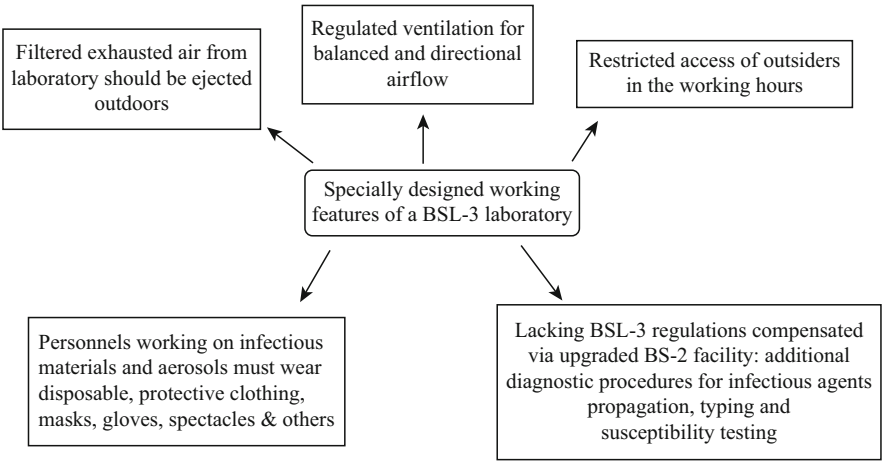

Fig. 5 Special provisions for a BSL-3 laboratory setup and the compensatory control via upgrading BSL-2 regulations

- The anti-room serves as a neutral zone, while the cell culture facility inside the laboratory premises is considered **a warm zone**.
- All BSL-3 laboratories should have specially designed and engineered salient features, not available in BSL-1 or BSL-2 facilities. Some characteristic requirements **include a properly sealed, air-conditioned cell culture facility with HEPA air filter and double-door access zone provisions**.
- BSL-3 laboratory involves working with infectious materials, in specially designed BSL-3 cabinets. **The conditions in BSL-3 are implemented by highly skilled and enlightened scientific investigators having long-term exposure and experience in working with infectious agents**.
- *Laboratory workers should be specially trained in handling highly pathogenic and potentially lethal agents* via *direct and regular supervision*.

Some specially designed and engineered features in the BSL-3 laboratory setup are as follows:

- The filtered exhaust air from the laboratory room is ejected into the outdoor environment.
- The ventilation in the laboratory is regulated to provide balanced, directional airflow.
- Access to the laboratory is restricted and minimized during work.
- All special practices and safety equipment for BSL-3 on top of recommended standard microbiological practices are rigorously followed.
- All laboratory personnel working with infectious materials and aerosols should wear **appropriate disposable protective clothing, mask, gloves, spectacles, and other necessary protective guards**.
- In the absence of proper BSL-3, the BSL-2 facility is upgraded with an advanced safety level by employing more safety measures such as diagnostic procedures involving the propagation of an identification agent, typing, susceptibility testing, etc. during routine work.

Biosafety Level 4

- ***Biosafety level 4 (BSL-4) is appropriate for working with dangerous, high-risk, and potentially harmful agents posing a severe life threat for which no vaccines and treatment are currently available.***
- The severe and fatal diseases may be contracted by BSL-4 agents even via inhalation of infectious aerosols. Thereby, BSL-4 norms mandate cautious handling in **highly restricted and containment laboratories.**
- BSL-4 agents include the highly lethal **coronavirus, Ebola virus, Lassa virus, Marburg virus,** and agents causing Crimean-Congo hemorrhagic fever, Bolivian and Argentine hemorrhagic fevers, and various hemorrhagic diseases.
- BSL-4 is also used while working with agents like the **smallpox virus, despite being** entirely eradicated and still being considered contagious enough, warranting additional safety measures.

- BSL-4 facility is specially built either in an entirely separate building or in a controlled area, isolated within a building. This provision is specially designed and manufactured with the use of negatively pressurized facilities for preventing contamination, which, if compromised, could severely aggravate an outbreak of aerosol pathogens.
- Building protocols include maintaining separate entrances and exits for the laboratory with **multiple shower rooms, a vacuum room, and an anti-room with ultraviolet light to inhibit the spread of infectious agents.**
- In the BSL 4 cabinet, the enclosure is gas-tight, and all materials enter and leave through a dunk tank or double-door autoclave. Class III cabinets are sometimes called glove boxes with gloves being attached to the front, preventing direct contact with hazardous materials. These custom-built cabinets are often arranged in a line with the laboratory equipment installed inside being built according to the requirement.
- A very strict and specific operations manual is followed for working in the BSL-4 facility. Safety measures are crucial and aptly designed to destroy and decontaminate even the traces of infectious biohazard materials.
- Multiple doorways with airlocks are fitted on the entrance(s) and exit(s) apart from being electronically secured to prevent both doors from opening at the same time and, hence, maintain a restricted air flow.
- All air and water service going to and coming out of a BSL-4 facility should undergo rigorous and similar decontamination procedures to eliminate the possibility of an accidental infectious agent outburst into the outside environment.
- By obligation, all BSL-4 workers should use **disposable, positive pressure personnel suits with specially ventilated air supply enabled via a life support system.**
- The BSL-4 laboratory workers should have **intensive and rigorous training** in handling extremely hazardous infectious agents to get acquainted with a proper understanding and significance of biosafety, special handling of biosafety standards and practices, the biosafety level or BSL hoods, special laboratory design, and its use for biosafety purposes. They should be trained and regularly monitored by direct supervision of experienced scientists. Access to the BSL-4 laboratory should be restricted to trained workers and mentor scientists, only.

In short, the BSL-4 includes the following cautionary tasks:

- Wear clothing dedicated to use while working in BSL-4.
- Wear personnel protective clothing safeguarding the whole body with adequate air supply and a positive pressure suit.
- Following work, decontaminate all materials before exiting the laboratory.
- Go directly to the showering room, and take a complete bath before complete exit from the laboratory.
- For comprehensive information read the following references: CDC Biosafety Cabinets (1995, 1999, 2000, 2008, 2009).

5 Maintenance and Service of a Biosafety Cabinet

Biosafety cabinets are strictly maintained regularly in the following ways:

- The HEPA filters have a limited life span. The airflow and specific HEPA filter capacities are closely monitored during maintenance. The filtered air flow rate is reduced with time, depending on the load and working conditions. As soon as flow-through becomes too low in old biosafety cabinets, an audible and visual alarm becomes functional. Subsequently, the old, used, and contaminated HEPA filter is changed right away by well-trained professionals using a strict **bag-in/bag-out** procedure.
- The biosafety cabinet is regularly decontaminated via saturating with formaldehyde (gas) after each servicing and relocation.
- UV lamp has a definite shelf life and their power decreases with time. To ensure a high degree of disinfection, an old UV lamp should be periodically replaced. The newly installed UV light facility should be cleaned regularly, to make sure it remains dust-free.

6 Care and Maintenance of Laboratory Areas

The mammalian cell culture is associated with the following problems:

- Contamination of the cell culture materials including cell culture medium with various microorganisms such as **mycoplasma, bacteria, fungus/yeast,** and **viruses**.
- Possible contamination of various bio- and chemical hazard molecules to the health of the persons working in the mammalian cell culture laboratory.

Considering the high value of cell culture products and the risk of losses from microbial contamination, *good laboratory practices* (**GLP**) and *good manufacturing practices* **(GMP)** must be followed in a mammalian cell culture laboratory (Johnson 1991; Burger 2002; Coecke et al. 2005).

For maintenance of the GLP and GMP, the following steps need to be taken:

Wear a cleaned personal protective cloth (lab cloth/disposable apron), lab footwear and if necessary, protective eyewear apart from observing the following cautions:

- **The first step** for working in a mammalian cell culture laboratory is to switch on the laminar flow UV light and laminar air flow through the HEPA filter at least half an hour before starting any work. Normal visible light must be switched off in course of UV sterilization. Just before starting cell culture, switch off the UV light and switch on the visible light.

  NB: UV light causes thymine dimer formation in the same DNA strand of the microorganisms that prevents the concurrent (e.g., bacteria) replication followed

by cessation of cell division. The visible light activates the photolyase enzyme within the microorganisms and subsequently breaks down the thymine dimer. Thus, in presence of visible light, UV light may not have any effect on bacterial replication.

- **The second step** is to wash the hands before resuming work with the culture. Repeat hand washing after completion of work.
- **The third step** is to wear sterile disposable gloves.
- **The fourth step** is to rinse the gloved hands using 70% ethanol.
- **The fifth step** is to clean all work surfaces including the floor of laminar flow with 70% ethanol just before starting the work as well as on completion of cell culture.
  NB: 70% alcohol kills the microorganisms.
- **The sixth step** is that before starting any cell culture work, one should make sure that every single material including cell culture containers, medium, etc. is sterile.
- Transfer of cells and other materials such as medium must be carried out inside the laminar flow hood only. Opening and closing of cell culture containers must happen within the laminar flow hood only.
- **The seventh step** is to check the aptness of incubator temperature, water, and CO_2 levels.
- **The eighth step involves** warming the medium to 37 °C using a water bath. Wipe out the medium bottle surface with 70% alcohol after it is taken out from the water bath.
- **The ninth step is not to** forget to switch off the UV light and turn on the vacuum pump (if necessary only) before commencing the work (EEC Directive 1990).

Now, works in the laminar flow hood can be initiated

6.1 Switching Off the Laminar Flow Hood

Certain steps must be taken to switch off the laminar flow hood in a proper way. The purpose of these steps is to make the laminar flow area a completely clean and germ-free environment for use by another person or another set of experiments later in the day. In addition, all the associated instruments used around the laminar flow area such as the vacuum pump and water bath must be cleaned and switched off, to increase their operational longevity.

Briefly, the following steps are taken:

- Switch on the vacuum pump, water bath, and inverted microscope.
- Discard liquid (after adding Clorox) and solid waste into the proper trash containers.
- Clean the laminar flow work area with 70% alcohol.
- Switch off the normal light, and switch on the UV for 15–30 min.
- Flush out the dead germs air from the laminar flow area by switching on the laminar flow for a few minutes.
- Switch off the laminar flow hood.

Additionally, the following steps are required for a contamination-free cell culture:

- No eating, drinking, or smoking inside the cell culture facility.
- No talking, or sneezing alongside while wearing or working with a disposable mask.
- Pipette aids may be used to prevent ingestion and keep aerosols down to a minimum.
- When working with human blood, cells, or infectious agents, appropriate biosafety level practices must be followed including post-culture cleaning and aseptic maintenance of the culture area. Generally, infectious human samples are used in a separate area, away from the main cell transfer area.
- *One should handle all patient samples as potentially biohazardous materials.*
- There must be a gap between working with infectious human samples and routine cell culture maintenance. During this time, the laminar flow must be UV sterilized and cleaned using 70% ethanol. This ensures an observation of **universal precautions** at all junctures!
- All biological wastes are autoclaved and destroyed.
- All liquid wastes are treated with bleach before being disposed of.
- Every 6 months, the cell culture laboratory must be **fumigated** for eliminating sporulating bacteria and fungus that may contaminate the air in the cell culture room.
- Replace the HEPA filter every 6 months or as per the regulatory schedule.
- Start with new stock every 3 months or as the passage number of cells required. Cells may genetically change in case of continuous culturing. In this context, follow ATCC guidelines.
- Before starting with a new culture vial, check for its mycoplasma contamination.

Cleaning of the cell culture laboratory instruments can be accomplished as follows:

- Every instrument used for the mammalian cell culture laboratory must be routinely cleaned with **disinfectants (mostly 70% ethanol)** to prevent undesired microbial growth.
- Water in the water bath must be changed after a couple of weeks apart from regular treatment of the water bath with **antibiotics/antimycotic agents**.
- Similarly, the water tray inside the CO_2 incubator used to maintain the moisture must be cleaned regularly, besides being **autoclaved** and freshly filled with sterile water.
- The CO_2 incubator itself can be cleaned regularly. Nowadays, a **programmable CO_2 incubator** is available which can automatically increase the temperature **just like a hot oven** and kill the microorganisms via **dry heating.**

  NB: Put the cells in an alternative place before dry heating the incubator.
- Similarly, the inverted microscope area must be cleaned with **70% alcohol** and disinfection agents to remove the contaminating microorganisms.

- Other agents for decontamination of the mammalian cell culture laboratory air from the fungal spores or bacteria or to decontaminate the biohazard containers may use formaldehyde, chlorine dioxide, and vapor phase hydrogen peroxide (Czarneski and Lorcheim 2011).

  NB: The CO_2 incubator and the microscope area have higher chances of contamination.

6.2 Plan for Scheduled Maintenance of Incubator

Every instrument has a manual, and the CO_2 incubator used in the mammalian cell culture incubator is no exception. In general, standard protocols as described in the incubator manual, are utilized for its maintenance. In general, the following steps should be taken:

- Replacement of gas inlet filters and HEPA filters every year as indicated in the manual.
- Fan and fan wheels should be cleaned every 6 months.
- Check whether the temperature is maintained rightly or not at least once a year using a NIST-certified thermometer.
- CO_2 levels should be checked every month.
- The water tray should be cleaned, autoclaved, and filled with fresh autoclaved water every week.

6.3 Disposal Management of Waste Generated from Cell Culture

- Mammalian cell culture waste is the source of growth of environmentally threatening microorganisms. Besides, cell culture waste may contain health-hazardous biomaterials and chemical compounds. Thus, it is a prerequisite for a novice or newcomer in a mammalian cell culture laboratory to learn about the disposal of cell culture waste. Different types of wastes may be disposed of as follows:
- Liquid waste including cell culture medium is inactivated by treatment with hypochlorite solution (**10,000 ppm)** for 2 h, following which it is disposed of in a specified sink.
- The solid waste contains polystyrene-grade cell culture containers (Petri plates/flasks), pipettes, tubes, tips, microfuges, etc. These solid wastes should be placed in heavy-duty sacks before incineration.
- Contaminated pipettes should be placed in hypochlorite solution (2500 ppm) overnight before being disposed of by incineration.
- Special licenses are mandatory for working on genetically modified organisms (**GMO**s). Separate sets of rules and regulations are required for waste disposal in a GMO laboratory.

  NB: For mammalian cell culture, the solid waste autoclave is generally not recommended.

7 Safety Equipment and Good Laboratory Procedures

Spillage of contaminated cells/microorganisms including that of air or floor is one of the possibilities while working in the mammalian cell culture laboratory. Accidents may also happen because of exposure to broken glass, needles, and contaminated infectious agents.

Prevention of contamination or any injury to the human body from broken glasses or used-up needles is the first priority of the mammalian cell culture laboratory. However, prevention of contamination is not the only requirement to be maintained in the mammalian cell culture laboratory.

- Before working in the mammalian cell culture laboratory, one must understand that like in any other research laboratory, chances of accidents may also be there in cell culture laboratories. There may be accidental spillage of chemicals/other agents to the eye or on the body or the laboratory floor and in the laboratory air.
- Fire may break out in the laboratory because of an electrical short circuit or other reasons.
- The safety of the laboratory personnel working in a mammalian cell culture laboratory is of utmost importance (US Department of Health, Education and Welfare 1974; US Food and Drug Administration Guidance 2007; US Department of Health and Human Services 2009; National Institute of Health Laboratory safety website).

The safety of the mammalian cell culture laboratory can be maintained by the following instruments:

1. Safety instruments specific for mammalian cell culture laboratory
2. Safety instruments common to any research laboratory

7.1 Safety Instruments Specific for Mammalian Cell Culture Laboratory

7.1.1 Laminar Flow Hood and Biosafety Cabinets

- The basic purpose of the laminar flow hood is to create a sterile environment so that mammalian cells could be handled and cultured without any contamination by microorganisms such as viruses, mycoplasma, bacteria, fungi, and others.
- The second most important use of a laminar flow hood is the protection of the laboratory personnel from infection with various airborne pathogens including viruses, mycoplasma, pathogenic bacteria, etc.
- To create a sterile environment in the laminar flow hood used UV light (260 nm wave length) that damages the DNA of pathogens by forming a thymine dimer.
- This kind of machine also uses a HEPA filter, through which only filtered and germ-free air can pass.

- Additionally, just before commencing the work, the floor of the laminar flow hood is generally swiped with 70% ethanol which destroys any bacteria that may be present inside the laminar flow hood because of multiple reasons.
- For proper utilization of the laminar flow hood and to work in a germ-free environment, the working area inside the hood must not be overloaded and the HEPA filter must be changed just before the expiry date.

7.2 Safety Instruments Common to Any Research Laboratory

Certain safety instruments are common to any research laboratory including biological, chemical, physical, or engineering.

Here is a list of such safety instruments:

First aid kits
Chemical spill kits
Fire extinguishers
Fire blankets
Chemical fume hood
Safety showers
Eyewash station
Refrigeration equipment
Flammable liquid storage cabinet
Safety cans and portable safety shield

7.2.1 First Aid Kits

Individual laboratories should have their first aid kits ready at a nearby location known to all. Laboratories can either buy individual components of the first aid kit or the complete first aid kit from a nearby **Red Cross** shop or another store. Supplies should be regularly checked, particularly on the last date before the drugs/medicines could be used. Read the manual of the kit. The outdated materials must be replaced from time to time.

A First Aid Kit May Contain the Following Materials

- A first aid kit is a set of materials and tools collected in a box (called a first aid box) that are used for emergency treatment of minor injuries or scrapes, burns, bruises, and sprains.
- A complete first aid kit is not only a collection of various materials but also contains a manual that describes the importance of various materials present in it with the process or steps of their usefulness.
- All the emergency phone numbers are listed in this kit so that in case of emergency, these could be contacted.
- Several general over-the-counter medicines such as acetaminophen and ibuprofen, hydrocortisone cream (1%), calamine lotion, antibiotic ointment, and an

antiseptic solution (like hydrogen peroxide), alcohol/antiseptic wipes or ethyl alcohol, and thermometer are present in a first aid box.
- Some other materials that could be present in the first aid kit are band-aids of several sizes, elastic bandages, sterile gauze pads of different sizes, adhesive tape, a splint, tweezers, sharp scissors, safety pins, disposable instant cold packs, plastic nonlatex gloves (at least two pairs), flashlight and extra batteries, a blanket, mouthpiece for giving cardiopulmonary resuscitation, etc.
- Following first aid treatment, the person may visit a doctor if the condition necessitates. In this case, the emergency phone number may be used.

7.2.2 Chemical Spill Kits

If a mammalian cell culture laboratory uses any hazardous chemical(s), it should have access to a chemical spill kit. Every laboratory personnel in the laboratory should know the content and location of the chemical spill kit.

Materials Found in Chemical Spill Kit

Absorbent materials such as pillows, pads, and several spongy clean surfaces may be present. Neutralizing materials comprise sodium bicarbonate for acids and citric acid for bases. Personal protective equipment (**PPE**) such as gloves, laboratory coat, splash goggles, dustpan, and broom to aid in the cleanup of solid spills are also important. A spill kit could be purchased from any vendor who supplies chemicals or laboratory safety materials.

7.2.3 Fire Extinguishers

Every laboratory including that of mammalian cell culture must have fire extinguishers. A fire extinguisher is a portable device that discharges a jet of water, foam, gas, or other materials to extinguish a fire.

Table 2 *presents the four kinds of fire extinguishers available.*

It is of utmost importance to the laboratory personnel to know the exact locations of all the fire extinguishers in the laboratory, along with the type of fires for which a particular fire extinguisher would be appropriate. The laboratory personnel must be trained in the process to operate a fire extinguisher, correctly. **The date of expiration of all fire extinguishers must also be noticed**.

Table 2 Description of varied fire extinguishers configurations

Type of fire extinguisher	Type of fire
Class A	Ordinary combustible materials (paper, wood, cardboard, most plastics)
Class B	Flammable or combustible liquids (gasoline, kerosene, grease, and oil)
Class C	Electrical equipment (appliances, wiring, circuit breakers, outlets)
Class D	Combustible materials often found in chemical laboratories (Mg, Ti, K, Na, etc.)

7.2.4 Fire Blankets

- Fire blankets are one of the important safety devices that are recommended for all laboratories utilizing flammable liquids. A fire blanket is made up of **fiberglass** or **Kevlar** and can be easily folded. A larger fire blanket is made up of **wool (sometimes treated with a flame-retardant fluid)**.
- A fire blanket can withstand as high as 900 °C and knock out the oxygen supply toward the fire.
- The main purpose of a fire blanket is to extinguish the accidental fire in the clothes of the laboratory personnel.
- Sometimes it may be useful to extinguish a small-scale fire in any laboratory instruments. However, in case of accidental fire in the clothing of the laboratory personnel, the **stop-drop-roll method** on the laboratory floor is used and a fire blanket is generally recommended as a last resort.
- A fire blanket is also recommended to keep the shock victims warm.

  NB: These blankets are usually mounted on vertical quick-release containers so that they can be easily pulled out and wrapped around a person having accidentally caught fire.
- Besides fire blankets, fire extinguishers should always remain in the research laboratories on standby.

Use of Fire Blanket

For a fire to burn, all three elements of the **fire triangle** must be present, i.e., **heat, fuel, and oxygen.** The purpose of a fire blanket is to **cut off the oxygen supply,** thereby putting it out. The user manual must be read and understood properly; as an alternative to which, there must be proper training of the laboratory personnel from the experienced fire personnel regarding the use of fire blankets.

7.2.5 Chemical Fume Hood

- Most laboratories that use chemicals, including volatile/inflammable materials (primarily solvents), need a chemical fume hood.
- While the width of the fume hood ranges from 1000, 1200, 1500, 1800, and 2000 mm, the depth varies from 700 to 900 mm, and the height ranges from 1900 to 2700 mm.
- If a laboratory works with extremely hazardous chemicals, a **glove box** must be added to the chemical fume hood. The main purpose of a glove box is to create a negative air pressure so that the operator should not be directly exposed to any type of chemicals.
- At an instant, up to three people can simultaneously work in a single fume hood.
- Composition-wise, a fume hood contains a movable glass window **(sash)** and internal lights with vapor-resistant covers. A chemical fume hood may be either **ductless** or **ducted**. Low airflow **alarm control panels** are common in chemical fume hoods. This type of hood is made in such a way that it is characterized as resistant to both chemicals and fire.
- Functionally, a chemical fume hood captures, contains, and expels chemical emissions.

- Additionally, while the slash is down, a chemical fume hood provides a protective barrier between laboratory personnel and chemicals or chemical processes.
- *Chemical fume hoods have the following usefulness:*

The **primary usefulness** is to protect the laboratory personnel from toxic gases that may be produced during various laboratory experiments.

- This type of hood protects the environment inside the laboratory.
- Laboratory product safety is another use of chemical fume hood.

The secondary use of these devices includes protection against explosion and spill containment *while working inside the chemical fume hood*.

7.2.6 Safety Showers

- A safety shower is a unit designed to take a bath (whole body including the head) for a steady supply of water flow for around 15 min.
- These kinds of showers are located in an easily accessible place.
- Every year, the water flow of the safety shower must be checked.
- If necessary, clothes may be removed before safety showers.

  NB: Water must not be directly splashed on the eye due to possible damage that the high-pressure flow may inflict.

7.2.7 Eyewash Station

- In every laboratory, emergency eyewash stations are necessary where biohazardous materials are frequently used.
- It is recommended that the eyewash station should be located a 100 feet away or a 10 s travel distance from every area of the laboratory.
- The purpose of an eyewash station is to clean the face and particularly the eye from particles, chemicals, and other substances immediately after the spillage happens in the nearby regions of the human body. Water flows at a sufficiently controlled level that a person's eyes remain open without any further injury.
- According to the American National Standards Institute (**ANSI**) rule, an eyewash station requires a minimum flow rate of **0.4 gallons/min**.
- In this context, Occupational Safety and Health Administration (**OSHA**) protocols may be utilized to properly use this station.

  NB: According to OSHA protocol [29 CFR 1910.151(c)], eyewash units should be activated **at least weekly** and inspected annually for compliance with the ANSI Z358.1 standard.
- For further reading go to the following website: **https://www.osha.gov**

7.2.8 The Equipment for Refrigeration

- Several laboratories use flammable and explosive chemicals, which may need to be refrigerated. Such chemicals cannot be stored in refrigerators that are specially made and designed for domestic food and other materials. Domestic refrigerators

do not have any capacity to withstand accidental ignition of inflammable vapors or explosive chemicals.

- As opposed to domestic refrigerators, the laboratory-safe refrigeration equipment (also called **flammable-safe** or **explosion-safe**) is intended for use in **high-hazard occupancies** where any kind of external ignition source must be eliminated.
- Explosion-proof refrigerators are **hardwired**, using electrical conduit, into the building's electrical service which is **devoid of standard three-prong electrical plugs**.
- All flammable liquids requiring storage in a cool environment should be stored in one of two approved refrigerator types.
- There should not be any vapor release due to the closeup of the plugs or container caps.

  NB: On the top of the laboratory refrigerators, it must be written what could or could not be stored in them.
- Conversion of refrigerators from domestic to laboratory settings for storing inflammable explosive chemicals is not recommended.

7.2.9 Cabinets for Flammable Liquid Storage

- A small number of flammable liquids may be stored around the working area, and separate sets of containers/cabinets are needed to store these, as they are necessary for experimental laboratory purposes.
- The storage place should be dry and cool.
- The purpose of these cabinets is to protect the contents inside from **arousing a fire** or at least delay any such casualty for at least 10 min so that all the laboratory personnel should get enough time either to douse the fire or leave the laboratory unhurt.
- In general, flammable liquids in quantities exceeding **10 gallons** in a laboratory must be stored in flammable liquid storage cabinets.
- It is recommended that around 60 gallons of flammable liquid per small container can be stored in a flammable liquid storage cabinet.
- All the **oxidizers** must be kept away from the flammable and combustible liquids and **stored separately**.
- **Acids** and **bases** must be stored in their dedicated storage cabinets, separate from flammable storage.
- For your safety, bonding and **grounding** should always be used while dispensing flammable liquids.
- Flammable cabinets may have **self-closing doors**.

7.2.10 Safety Cans and Portable Safety Shield

Safety Can

- Safety Can is a type of container which is used to store **flammable liquids such as gas/oil mixtures, gasoline, or other flammable/combustible liquids.**

- This kind of container has a spring-closed lid, spout cover, and flame arrestor, designed with the intent to safely relieve the internal pressure. Safety cans must be compatible with the chemicals being stored.
- Safety cans are divided into two groups, namely, types I and II.
- While type I safety cans only have **one opening through which both pour and fill functions are maintained, the type II safety cans have two openings, one each for pouring and filling**. This fill opening serves as a vent when pouring.
- In general, a safety can have a 5-gallon capacity.

  NB: A safety can with damaged parts such as corroded spark arrestor screens or insufficient springs must be taken out of service and replaced immediately.

Safety Shields with Portable Capacity

- These kinds of containers can provide limited protection against chemical splash hazards, fires, and explosions.
- Such shields are in use, as and when a chemical hood sash does not work properly.
- Portable safety shields do not protect the sides and back of equipment.
- Therefore, this kind of shield works best if used in conjunction with a fume hood.

  NB: Laboratory equipment/chemical apparatus should be shielded on all sides so that there is no line-of-sight exposure to laboratory personnel.

Besides the above instruments and materials, the following additional materials are necessary for the safety of persons in a mammalian cell culture laboratory.

Protective Equipment for Personal Use

Several personal protective types of equipment are available that help laboratory personnel from direct contact with various chemicals/biological (e.g., microorganisms) spillage.

Some of the important examples are as follows:

- Disposable aprons and coveralls
- Heavy neoprene gloves and disposable vinyl gloves
- Face shield and goggles

Materials for Clean Up

In general, every laboratory has the following materials for clean-up:

- One empty 5 gallon plastic bucket with a lid for absorbent residues
- Plastic bag (30 gallons, 3 ml thick)
- Plastic dustpan and scoop

Additional Materials for Safe Laboratory Use

Mercury Spill Kit
Hydrofluoric Acid Antidote Gel-Calcium Gluconate

Safe Laboratory Practices

To ensure laboratory safety, safe laboratory practices should be practiced, as follows:

- All laboratory personnel must be trained well in mammalian cell culture procedures before independently working in the laboratory.
- Read all instrument manuals and research protocols before the formal commencement of any research task.
- Know about all the biological and chemically hazardous materials in the laboratory.
- Know the exact location of all safety instruments.
- Learn what to do in various emergencies.
- Wear a lab coat and protective equipment as appropriate.
- The use of ice from laboratory ice machines for eating purposes should be avoided.
- The use of laboratory ovens and refrigerators for personal food items should be avoided.
- The use of contact lenses around chemicals, fumes, dust particles, or other hazardous materials should be avoided.
- Inform supervisor and make a daily laboratory logbook for work.
- Avoid aerosol generation.
- In general, small kids are prohibited in the laboratory without a proper chaperon by the seniors.
- Every single chemical spill anywhere inside the laboratory must be cleaned/decontaminated immediately.
- Needles, blades, and glasses should be carefully handled, and used-up needles and blades or broken glasses should be kept in specified containers for future disposal in a proper way.
- Eating, drinking, smoking, and mouth pipetting must be avoided in a cell culture laboratory.
- Since dry ice can produce elevated carbon dioxide levels, avoid using dry ice in enclosed areas.
- Frostbite may happen if dry ice is mixed with isopropanol or ethanol.
- Hallways, corridors, and exit ways must be kept clear. Do not situate (even temporarily) laboratory equipment or supplies in these areas.

Basic ideas about the cell culture laboratory, instruments needed, and mammalian cell culture protocol can be found on the following web pages:

- https://www.atcc.org/~/media/PDFs/webinars/Presentations/2017/Cell%20Culture%20101.ashx
- https://www.vanderbilt.edu/viibre/CellCultureBasicsEU.pdf
- https://wikisites.mcgill.ca/djgroup/images/4/41/Cell_culture_manual.pdf
- https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/419/453/d5523for.pdf
- https://www.roswellpark.org/sites/default/files/sexton_3_10_16.pdf

- Aldrich/General_Information/1/fundamental-techniques-in-cell-culture.pdf
- https://www.ptglab.com/media/4457/the-complete-guide-to-cell-culture.pdf
- https://www.ptglab.com/support/cell-culture-protocol/cell-culture-protocol

8 Conclusions

This chapter describes the smooth and proper development of a mammalian cell culture laboratory and the associated necessary steps required for contamination-free safe culture of the mammalian cells as well as maintenance of safety of both the laboratory and the concerned researchers. The mammalian cell culture facility is mainly subdivided into the main laboratory, an anti-room for prevention of contamination, and an outer laboratory. The main laboratory contains a sterile laminar flow hood/biosafety cabinet for aseptic transfer, a sterile CO_2 incubator for the culture of cells, and microscopes such as an inverted microscope to observe the living status of cultured cells. The outer laboratory is normally divided into a general washing area; sterilization, media preparation, and storage area; and observation/data collection area.

Apparently for the establishment of a mammalian cell culture, laboratory **biosafety** is one of the most important aspects which needs to be considered. Starting from minimum to maximum biohazards, four biosafety levels, i.e., biosafety levels 1–4, are designated. While in general most mammalian cell culture laboratories are either biosafety level 1 or biosafety level 2 depending on mild or moderately potential biohazards, biosafety levels 3 and 4 are mainly required for highly infectious and pathogenic species of microbes. Since contamination of mammalian cell culture is a major hurdle, the next aspect of designing a mammalian cell culture laboratory is to prioritize **care and maintenance of the whole area to create a germ-free environment**. Finally, the **safety features of both the cell culture laboratory and laboratory personnel** need to be planned systematically. Safety of the mammalian cell culture laboratory can be maintained by two types of equipment: (1) **safety instruments specific for mammalian cell culture** (laminar flow hood/biosafety cabinets containing HEPA filters and UV light) and (2) **safety instruments common in any research laboratory** (first aid kits, chemical spill kits, fire extinguishers, fire blankets, chemical fume hoods, safety showers, eyewash stations, refrigeration equipment, flammable liquid storage cabinet, safety cans, and portable safety shield). All the necessary instruments needed to develop a mammalian cell culture laboratory are additionally described in this chapter.

9 Cross-References

► Culture of Continuous Cell Lines
► Culture of Neuron and Glia Cells
► Isolation and Primary Culture of Various Mammalian Cells
► Primary Culture of Immunological Cells
► Stem Cell Culture and Its Applications

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Mammalian Cell Culture Laboratory: Equipment and Other Materials

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https://doi.org/10.1007/978-981-19-1731-8_3-1

Abstract

This chapter describes all the instruments necessary to work in a cell culture laboratory safely and effectively, including the maintenance and propagation of cultured mammalian cells. In a well-designed and established mammalian cell culture laboratory, the main cell culture facility is equipped with laminar flow hood/biosafety cabinets, CO_2 incubator, inverted microscope, and other accessory grade small instruments such as temperature-controlled tabletop centrifuges, hemocytometer, or automated cell counter. All these instruments are necessary for mammalian cell culture and can be maintained safely and easily. The anti-room is generally located just outside the main cell culture laboratory which is a buffering zone before entering the main cell culture laboratory to limit the dust particles and microorganisms inflow from the laboratory personnel. This anti-room may be utilized for keeping shoe racks and disposable personal protective equipment (**PPE**) or accessories. Here, laboratory personnel should wear laboratory dress codes and disposable protective clothes before entering the main cell culture laboratory. A HEPA filter room and a shower room are also located adjacent to the anti-room. The outer cell culture laboratory should be generously equipped with various instruments related to hot moisture/dry heat sterilization (autoclave/oven, etc.) and washing (big sinks), medium preparation (chemical storage self, balance, refrigerators, water bath, water purification system, etc.), maintenance of cultured cells(−20 °C and −80 °C deep freezers, liquid nitrogen containers, etc.),

and storage racks of sterile, disposable cell culture containers (plate, flasks, tubes, pipettes, etc.). Various general safety instruments (e.g., eye wash station) and biohazard disposal containers are also assigned space in this outer cell culture laboratory. *This chapter briefly narrates all these instruments and their usefulness.*

Keywords

Laminar Flow hood/ Biosafety Cabinet · CO_2 Incubator · Microscopes · Cell Counters · Hemocytometers · Cell Culture Containers · Transfer Aids · Membrane Filters · Cell Culture Centrifuges · Sterilization Instruments: Autoclave and Oven · Refrigerators/ Freezers/ Deep Freezers · Liquid Nitrogen Containers · Biohazards Waste Containers

1 Introduction

A mammalian cell culture laboratory is equipped with various instruments needed for several direct and indirect activities. As described in chapter ▶ "Establishment of a Cell Culture Laboratory," the main cell culture laboratory is equipped with a laminar flow hood (LFH) or biosafety cabinet, a CO_2 incubator, a cell counter, and one or more microscopes. Additionally, bench-top low-speed centrifuges are kept away toward the back of the main laboratory premises. Other accessory instruments are placed **outside the laboratory's cell culture facility**. These include sterilizing oven and autoclave, water bath, water purification system, refrigerators, −20 °C and −80 °C deep freezers, liquid nitrogen containers, biohazard waste containers, and disposal provisions. There should be storage units for keeping sterilized culture vessels, accessory sterile glassware, and plastic containers.

The main cell culture laboratory is **the cleanest area** where **transfer of cells and medium** (in the laminar flow hood), **observation** (through microscopes), **counting** (through automated cell counter or hemocytometer), and **culture** (through CO_2 incubator) of mammalian cells could be done. Low-speed centrifuges present at the back of the central space are used to pellet down the cell suspension.

Out of the various outer laboratory instruments, refrigerators are used for culture media **storage**; freezers are used for storing the temperature-sensitive **reagents** such as trypsin-EDTA; deep freezers are used for storing the **cells** and **some chemicals**; **and** liquid nitrogen containers are used for the **permanent cell storage**. The purified and deionized water generated from the purification system is utilized for medium preparation, catering multiple utilities. The water bath is used for thawing the frozen cells, warming up stored cell-culture medium and reagents besides incubation of various cell culture reactions at different temperatures.

Since in a cell culture laboratory every single glassware, plasticware, and other usable material must be sterile, autoclave and dry-oven utilities are of immense significance. Of note, while an autoclave kills the microorganisms through moist heat and high pressure, a dry oven eliminates the microorganisms through **dry heat**.

Finally, waste containers are used for storing and time-to-time disposal of biohazardous wastes produced during cell culture. It is highly essential to understand not only the various instruments used in the mammalian cell culture laboratory but also the working of these instruments to identify various important parameters for culturing the mammalian cells (Skoog 1985).

Here is a brief discussion of instruments needed for the mammalian cell culture facility.

2 Laminar Flow Hood and Biosafety Cabinet

A **laminar flow hood (LFH)** is an aseptic working station equipped with a UV lamp for sterilization and a quality **HEPA** filter for air filtration. It provides a sterile atmosphere to perform mammalian cell culture (Favero and Berquist 1968).

The sterile environment within an LFH is maintained in the following manner:

- UV light arrests bacterial growth by inhibiting their replication (*it forms a thymine-thymine dimer in the same strand of DNA*).
- Air is passed through a HEPA filter to maintain a sterile and clean environment after 99.99% removal of >0.3 μm particles, including the microbes such as bacteria, inside the laminar flow chamber. The HEPA-filtered air quality meets at least the Class 100 Clean Standard 209D and maintains a (90 ± 20%) fpm flow, at static (0.6–1.2) atmosphere pressure.

  NB: Mycoplasmas and viruses can easily pass through the HEPA filter because of their smaller size.
- In an LFH, HEPA-filtered air flows outward and circulates in a way that the UV sterilized microbes present inside the hood are ejected.
- Therefore, when the laminar flow is kept in the **ON** position, the sterile air flows inside and aids in maintaining the hygienic environment necessary for mammalian cell culture.
- Further, the working area of LFH is sterilized by wiping the surface with 70% ethanol.
- Thus, an LFH not only protects the cultured mammalian cells from microbial (e.g., fungi/molds or bacterial) contaminations but also safeguards the users from potential infection risk (Fig. 1).

Depending on the direction of air flow, laminar hoods are of the following two types:

1. Vertical laminar flow hood
2. Horizontal laminar flow hood

Here is a brief description of them:

Fig. 1 Front view of a stainless steel laminar air flow (LAF)

2.1 Vertical Laminar Flow Hood

- In a vertical LFH, the **air is pushed vertically downward at a constant speed through a HEPA filter located at the top.**
- Vertical LFH has a pivoting transparent plastic or glass sash **providing a physical barrier, as user protection** (Fig. 2).
- Vertical hoods are used while working with biohazard material, toxins, or radioactive chemicals.
- This kind of LFH is regularly used in microbiology, immunology, stem cell research, vaccine production, forensics, and many other biotech and pharmaceutical industries.

2.2 Horizontal Laminar Flow Hood

- In a horizontal LFH**, the air is directed at a constant speed through a HEPA filter backward toward the frontal horizontal work surface**.
- In general, horizontal laminar flow hoods are taller and have a larger working area than their vertical counterparts.

Fig. 2 Distal view of a laboratory-scale vertical laminar airflow

- **The absence of a sash in horizontal LFH** allows more space for placing dissection instruments, such as microscopes or cell vortex spinner (Fig. 3).

2.3 Basic Guidelines When Using a Laminar Flow Hood

1. The UV lamp is turned on for at least 15–30 min, with visible light and laminar air flowing off mode.
2. When the UV lamp is turned off, both laminar airflow and visible light are turned on.
3. The glass shield is moved to the marked level followed by inward blowing of sterile air for **about 10 min to saturate and purify the inside space**.
4. The inside of the hood and all the instruments are wiped well with 70% alcohol.

Fig. 3 Side view of a laboratory-scale horizontal laminar airflow

5. The inside of the hood is always kept clean and organized. All spills are immediately wiped with 70% alcohol.
6. All work should be done in the middle of the hood while being careful not to block the front area, housing the air filter.
7. While transferring the cells or medium, the tip of the pipette or the mouth of the cell culture container should not touch the wall of LFH.
8. After finishing work, the LFH surface should be wiped again with 70% alcohol, before switching off the blower and finally closing the hood.
9. LFH should not be used as storing place for medium, cell culture containers, etc. which may not only block proper air flow but could also aggravate the deposition of dust, microbes, etc. (Jain and Reed 2019).

2.4 Basic Differences Between a Laminar Flow Hood and a Biosafety Hood/Cabinet

In an LFH, unfiltered exhaust air blows out to the environment causing risk to the laboratory personnel and the environment itself, whereas in a biosafety cabinet, all the exhaust air is HEPA filtered to remove harmful microbes, thereby not posing any environmental concern.

NB: Biosafety cabinets and various biosafety levels (levels 1–4) are already discussed in chapter ▶ "Establishment of a Cell Culture Laboratory."

3 Carbon Dioxide Incubator

- A CO_2 incubator is an essential requirement for growing live mammalian cells in a cell culture laboratory (Fig. 4).
- Live mammalian cells are grown in proper cell culture medium within cell culture containers (Petri plates/flasks, etc.), incubated at 37 °C.
- All CO_2 incubators are water-jacketed and maintained at 37 °C in a sterile (HEPA filtered) air environment enriched with 5% CO_2.
- This CO_2 amount is considered optimum for maintaining the propagation-suited pH of mammalian cells in culture. In the case the incubator is not water-jacketed, freshly autoclaved Millipore (**Milli Q**) water is kept in a sterile container to maintain 95% humidity.
- To avoid microbial contamination, periodic thermal decontamination should be enabled through programmed auto-heating.
- The shelves should be regularly autoclaved and wiped with 70% alcohol to avoid any microbial contamination.
- Thus, sterile CO_2 incubators provide a suitable physiological environment for mammalian cell growth (Tamaoki et al. 2002a, b; Triaud et al. 2003).

Fig. 4 Front view of a CO_2 incubator

3.1 CO_2 Incubators Maintain the Cell Culture Environment Artificially with the Following Functions

3.1.1 Maintaining the Physiological Temperature of the Culture Medium

The temperature in the cultured cells in a CO_2 incubator is maintained at around 37 °C throughout.

3.1.2 Maintaining Humidity of the Culture Medium

At 37 °C, the culture medium could get dried up, owing to the increased metabolic activity of the cells. To prevent this, HEPA-filtered air is saturated with water using either water-jacketed boundaries or sterile water kept inside. Generally, humidity inside the LFH is around 95%.

3.1.3 Maintaining pH of the Culture Medium

The pH of a mammalian cell cytoplasm is 7.2 and that of blood is 7.4. Thus, the pH of the cell culture medium is maintained between 7.2 and 7.4. However, the metabolic activity of cells increases the medium pH, making it alkaline. The supplied CO_2 reacts with water in medium (H_2O) to form H_2CO_3 (a weak acid), providing $H^+ + HCO_3^-$.

$$H_2O + CO_2 \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^-$$

The generated H^+ neutralizes the excess alkali owing to the metabolic activities of the cultured cells, restoring the pH of the culture between 7.2 and 7.4.

Thus, the ***CO_2 in a CO_2 incubator acts as a buffer***.

The culture medium has a **phenol red indicator** which changes color with pH variation. ***In the acidic state, phenol red becomes yellow, while in the alkaline state,***

Fig. 5 Schematic view of a pH meter

it becomes pink. If CO_2 is too high, the medium becomes acidic (**yellow**), while if it is low, the medium becomes alkaline (**purple**) (Fig. 5) (Cheng and Zhu 2005).

4 Microscopes

Microscopes are essential equipment for a cell culture facility and are used to see both live and dead cells. There are many types of microscopes, i.e., phase contrast, bright-field, inverted, etc. **Nonsterile cells** can be seen either in the live or dead state on slides or cell counter/hemocytometer using regular phase contrast and bright-field microscopes, whereas **sterile cells** in the culture are visible only under **an inverted microscope**. For the application of cell cultures, **3D imaging** of the cells using a **phase-contrast microscope** plays a critical role. Out of the various microscopes and their application for the routine day-to-day activity of maintaining sterile growth of live mammalian cells, the ***inverted microscope is the best choice for routine mammalian cell culture***.

Besides **inverted microscope**, **phase contrast**, **bright-field,** and **confocal microscopes** have varied utilities in mammalian cell culture. So, in the following paragraphs, all these microscopes have been briefly discussed (Brenner 1985).

4.1 Inverted Microscope

- In 1850, the inverted microscope was discovered by *John Lawrence Smith* to observe the sterile cells in culture which can't be viewed in a sterile culture flask or plates using phase contrast or bright-field microscopes (Smith 1852; Meyer et al. 1980).
- An **inverted microscope** is similar to a compound microscope, except for the fact that the **components are placed in inverted order**. In a normal microscope, the condenser lens and the light source remain below the stage and the specimen. However, in an inverted microscope, the condenser lens is positioned above the specimen. The light source would be kept just above the condenser lens from which light is illuminated on the specimen across upside instead of the bottom. The objective lens is present below the stage which collects the light from the condenser, magnifies the image, and sends it to the ocular lens. The light is reflected from the ocular lens via a mirror to view the erect image of the specimen (Fig. 6).
- These inverted microscopes can be fitted with various accessories, such as still and **video cameras, fluorescence illumination, confocal scanning, and many other probes.**
- Inverted microscopes are used for visualizing cells in sterile cultures without disturbing the sterility.

- The cells in the culture flask or plate remain at the bottom while the medium floats at the top. This type of microscope is suitable for viewing culture vessels such as **Petri dishes** or T flasks.
- **An inverted microscope through which live mammalian cells are observed can be configured for the completion of the following tasks:**
  - Detection, identification, intracellular localization, and quantification including colocalization of specific proteins using fluorescently labeled antibodies.
  - All the observations via inverted microscopes can be enhanced and analyzed through videography including differential interference contrast (**DIC**) at a high resolution.
  - Electrophysiological works, particularly used in patch clamp technique.
  - Helps in assisted reproductive technology (**ART**) such as intracytoplasmic sperm injection (**ICSI**), in vitro fertilization (IVF), and intracytoplasmic morphologically selected sperm injection **(IMSI)** and finally used for micromanipulation.

4.1.1 Guidelines for Use of Inverted Microscope

- Wipe the nonmovable stage with alcohol before cleaning the lenses with lens paper.
- Place the culture plate or flask of cells on the stage.
- Turn on the light.
- Turn the objective lens turret to the smallest magnification (4×).
- Turn the turret to a higher magnification. A 10× lens is usually sufficient for routine work.
- Cells can be viewed using the ocular lenses while moving the flask slightly and focusing to an optimum separation.

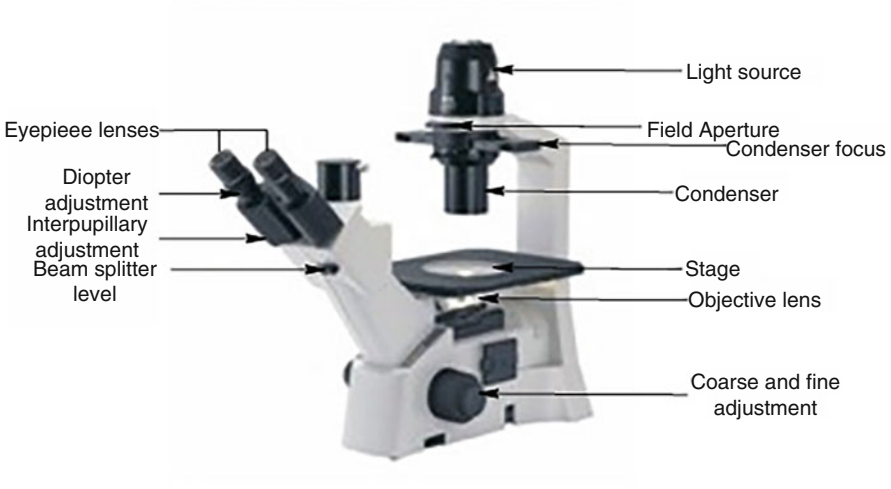


Fig. 6 Description of an inverted microscope

- The focus knobs are adjusted for the best possible resolution. To focus both eyes, the focus knob is focused first with the right eye closed. Then close the left eye and the focus knob is focused by turning the right ocular lens.
- Phase rings help to get a clear image of some cell types. So, a phase ring is moved back and forth, in the light direction for a better observation.
- After work, the stage is wiped off with alcohol, before cleaning the lenses and turning off the microscope.

4.1.2 Applications of Inverted Microscope

- Inverted microscopes can be used to observe the living cells and tissues present at the bottom of any culture vessels such as flask, Petri plate, etc. without preparing the slide, in a natural state.
- The microscope is also used in certain diagnostic assays such as MODS (*microscopic observation of drug sensitivity*) assay.
- Experiments done on microfluidic slides can be observed under the inverted microscope in real time when connected to a video camera (Maziarski 1988).

4.2 Bright-Field Microscope

Bright-field microscopy is the most elementary form of microscopic illumination technique that is generally used with compound microscopes. The terminology "bright field" infers the logic that the specimen being viewed in this technique is generally dark and contrasted by the surrounding bright-viewing field. Sometimes, simple light microscopes are also referred to as bright-field microscopes (McNamara 2005; Liu et al. 2014).

4.2.1 Invention of Bright-Field Microscope

- A typical bright-field microscope could be considered as a modification of a compound light microscope, for which major credit goes to *Zacharias Janssen* of Holland in 1595.
- The contribution of ***Ernst Abbe*** (1873) also deserves a mention in this context who is credited with improving the oil immersion objectives and optimizing the illumination device. Subsequently, ***Koehler*** illustrated a mechanism for improved sample illumination which remains accepted even to date.

4.2.2 Working Mechanism of Bright-Field Microscope

- Image formation in a bright-field microscope is attributed to a magnification of the light by the objective lens which further transmits it to an eyepiece and subsequently to the user's eyes.
- The source of sample visualization is the marginal absorption of light by stains, pigmentation, or dense areas of the sample which manifests the contrast herein.
- In bright-field microscopy, the vicinity conditions are bright while the object is dark. The objects are dark because of their scattering nature, such as endogenous pigments or exogenous dyes.

- Light from the source is transmitted along the direction of the optical axis through the sample into the objective lens. This is beneficial for specimens with good scattering ability or which are naturally or synthetically colored.
- As the light encounters the specimen, its intensity is reduced from that of the surroundings, resulting in a darker appearance.
- Based on light source positioning, two working modes are recognized, namely, transmitted and incident illumination. In the transmitted mode, the illumination source is exactly below the sample for an upright microscope. In general, light passes through the sample only once on its way from the source to the objective.
- Kohler illumination is the standard configuration for transmitted mode, a constant feature of almost all biomedical microscopes. Nevertheless, since the condenser can be vertically and laterally moved, the user intends to adjust the condenser field aperture focus and numerical aperture setting.

4.2.3 Usage Specifications of Bright-Field Microscope

- Bright-field microscopy is best suited to view stained or naturally pigmented specimens such as stained slides of tissue sections or living photosynthetic organisms.
- Illumination of a bright-field microscope is most suitable for a sample having an intrinsic color, e.g., chloroplasts in a plant cell. Magnification in a bright-field

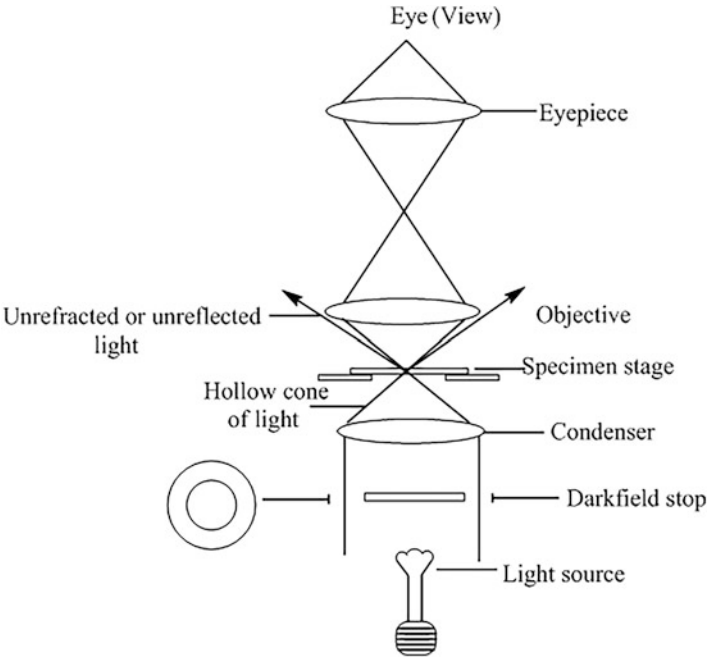


Fig. 7 Working module of a bright-field microscope

microscope is limited by the resolving power possible with the wavelength of visible light.
- The technique is not suited for viewing live bacterial specimens and is rather inept for nonphotosynthetic protists or metazoans or unstained cell suspensions or tissue sections (Fig. 7).

4.2.4 Sample Imaging in a Bright-Field Microscope

The step-by-step procedure to view a sample using a bright-field microscope is as follows:

- Position the specimen on the stage, and the incandescent light from the light scope is aimed at a lens beneath the specimen. This lens is called a condenser.
- The condenser lens is equipped with an aperture diaphragm that controls and focuses the light on the specimen. In short, light passes through the specimen and is then collected by an objective lens, situated above the stage.
- The light from the source first enters the condenser which magnifies and concentrates it through the objective onto the eyepiece or user's eyes.
- For good results with this technique, the microscope should have a light source capable of providing intense illumination necessary at high magnifications and lower light intensities for lower magnifications.
- Different magnifications are ideally suited for different samples. For example, stained bacteria are viewed at 1000×, thick tissue sections could be viewed at 100× and 400×, thin sections with condensed chromosomes are observed at 1000×, and large protists are distinctly observed at 100×.
- Living preparations (unstained wet moths) and pond water are visualized at 40×, 100×, and 400×; living protists are observed at 40×, 100×, and 400×; and algae and other microscopic plant materials are viewed at 40×, 100×, and 400×.
- In general, smaller specimens are difficult to observe without any distortion, particularly when there is no pigmentation.

4.2.5 Maintenance Cautions During Use of Bright-Field Microscope

For upright and regular working of a bright-field microscope, the following aspects must be regularly checked and corrected whenever needed:

- Regular cleaning of oculars and objectives. Do not ever use paper towels or other rough paper products for cleaning. Prefer either 70% isopropyl alcohol or dilute methanol. For stage and base cleaning, mild detergent and soft cloth can be used.
- Avoid damaging oculars and remaining optical parts with eye makeup or other rubble.
- Handle carefully to avoid random and arbitrary movements.
- Take caution such as exposure to direct sunlight, high temperature, humidity, dust, and vibrational shockers.
- Use neat, clean, and stable materials to clean the lenses. Always prefer the first use for cleaning.

- Whenever not in use, the instruments should be covered with vinyl or plastic dust cover.
- While cleaning the oculars and objectives, the lenses and filters must be carefully removed only one at a time.
- Undue abrasion can damage the iridescent coating on the lens and, therefore, must be avoided.
- Always prefer not to take the eyepiece or objectives apart.
- While removing the microscope bulb, the instrument must be unplugged and the bulb should be allowed to cool. Subsequently, position the microscope on its side, open the bulb house, and remove the bulb using tissue paper. Use tissues to ensure that no residual fingerprints remain while picking up the new bulb. After inserting the new bulb, do not forget to close the bulb house.
- While setting the Koehler illumination, operate the field diaphragm and condenser diaphragm with utmost care. Move up the stage to its highest position and adjust the oculars for interpupillary distance so that only one light circle remains visible. Close the field diaphragm halfway and focus the smear at 10×. Similarly, ensure that the diameter of the illuminated image is smaller than the field of view. Always lower the condenser through the positioning knob steadily till a sharp and focused image of the field diaphragm edges is obtained. Adjust the condenser using centering screws so that the light circle is centered before opening the field diaphragm until the illuminated image is just larger than the field of view. Use the transformer in case more light is needed. Now, the Koehler illumination is set, making it mandatory to avoid any vertical motion of the condenser henceforth.

4.2.6 Advantages of Bright-Field Microscope

- Bright-field microscope is easy to use with nominal optimizations required for sample viewing.
- Some specimens could be viewed without staining, whereby the optics used produce an unaltered specimen color.
- The technique is adaptable with new technology and optional instrumental domains could be exercised via bright-field illumination for the versatility of accomplishable tasks.

4.2.7 Limitations of Bright-Field Microscope

- The requirement of an aperture diaphragm for contrast above a limit induces a likely risk of image distortion. This hurdle could be resolved via an iris diaphragm.
- The technique can't be used to observe live bacterial specimens, although when fixing, bacteria have an optimal viewing magnification of 1000×.
- The technique has very low contrast because most cells mandate staining before being viewed. Staining may interfere with interpretation *by* introducing extraneous details into the specimen.
- Finally, the technique requires a strong light source for high magnification, but intense lightning can produce heat that may be fatal for living organisms or tamper with the specimens.

NB: Visualizing living cells using bright-field microscopy often remains inconclusive as the unstained living cells hardly absorb any light. This is the major reason for nearly invisible cells in a bright-field microscope.

4.3 Phase Contrast Microscope

Experimentally, as light passes through cells, a small but invisible shift of light occurs. In phase contrast microscopy, these phase shifts are converted into amplitude variations. In the next step, the representation of these changes in amplitude as image contrast takes place as brightness variations are visualized through this type of microscopy. Thus, phase contrast is used to enhance the contrast of light microscopy images of transparent and colorless samples.

4.3.1 Invention of Phase Contrast Microscope

- The technique was unveiled by Dutch physicist *Frits Zernike* in 1934, through the discovery of a method wherein phase differences can be transformed into amplitude differences.
- The configuration invented by *Zernike* is presently referred to as positive or dark phase contrast, whereby the cell constituent to be examined appears darker than the surrounding background.
- *Zernike* was *awarded the 1953 Nobel Prize* for his rigorous work to simplify the distinct view of cell organelles and other subcellular structures.

4.3.2 Constitution of Phase Contrast Microscope

- The design modifications for converting a light microscope configuration to a phase-contrast mode are reasonably simplified and inexpensive.
- Apart from all the basic parts of a light microscope, a phase contrast microscope requires an annular phase plate and an annular diaphragm. The basic functions of these two are as follows.

The annular diaphragm encompasses the following features:

- It is situated beneath the condenser lens.
- It comprises a circular disc carrying a circular annular groove.
- The light rays are made to fall across the annular groove, after which they reach the sample to be observed.
- The image is processed at the back focal plane of the objective.
- The annular phase plate is positioned at this back focal plane.

The following are the important functions performed by the phase plate:

- It can be a negative or positive phase plate respectively comprising of a thick circular area and a thin circular groove.
- The thick or thin area in the phase plate is called as conjugate area.

- The phase contrast is obtained with the aid of an annular diaphragm and phase plate.
- This is estimated through the separation of direct rays from diffracted rays.
- While direct light rays pass through the annular groove, the diffracted rays move across the region outside the groove.
- The development of distinct contrasts in the view of distinct biological samples in this microscopy is due to their characteristic refractive indices.
- Caution must be exercised for the condenser annulus and phase rings to equilibrate with diameter settings and facilitate optical conjugation.

4.3.3 Working Principle of Phase Contrast Microscope

- Phase contrast microscopy decodes small phase changes to amplitude (brightness) variations, which are subsequently seen as image contrast distinctions.
- The specimens with null light absorption are known as phase objects. Such terminology is practiced as these regimens change the phase of light diffracted by them. Usually, there is a one-fourth phase shift of wavelength concerning background light.
- Our eyes are unable to detect these slight phase distinctions as these are not in the order of incident light frequency and intensity.
- The formation of high contrast images by a phase contrast microscope is due to cumulative increments in the phase differences between the incident and diffracted light.
- Enhancement in the difference of light phases is accomplished via slowing down the background light using one-fourth of a wavelength, with a phase plate just before the image plane.
- Upon focusing the light on the image plane, the diffracted and background light signals interfere destructively or constructively, resulting in decreased or increased brightness of sample-containing areas. Such mode of working results in enhanced or reduced brightness of the areas comprising the sample compared to that of the background light.
- Initially, light from a tungsten-halogen lamp passes through the condenser annulus in the substage condenser before reaching the specimen. This facilitates the sample illumination using the defocused parallel light.
- The specimen's nature plays a key role in determining the specific extent of image contrast. This is so as the light which asses through the specimen is not diffracted, forming a bright image on the rear aperture of the objective. Contrary to this, the light waves that are diffracted by the specimen pass the diffracted plane and focus only on the image plane. Such an arrangement separates background and diffracted light.
- The phase plate in the arrangement alters the background light speed by a complementary factor of one-fourth wavelength. Upon being focused on the image plane, the diffracted and background light could interfere constructively or destructively. This alters the brightness of sample-containing areas compared to that of background light. Many times, the background is dimmed by 60–90% using a gray filter ring (Fig. 8).

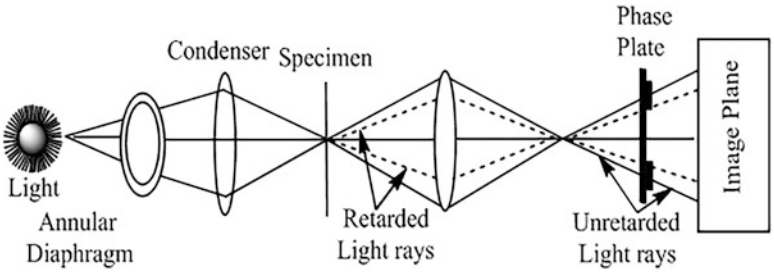


Fig. 8 Working module of a phase contrast microscope

4.3.4 Advantages of Phase Contrast Microscope

- The module allows living cell visualization in their natural state without prior fixation or labeling.
- The technique enables higher visibility of a highly transparent specimen.
- No sophisticated preparation involving sample staining is required to study an object, thereby saving a lot of time.
- The technique facilitates the visualization of living cells' intracellular components at a relatively high resolution. For example, the dynamic motility of mitochondria, mitotic chromosomes, and vacuoles is the basis of their distinct recognition.
- Phase contrast constituents can be configured to any bright-field microscope, subject to the phase objective synchronization with tube length parameters. Thereafter, the condenser accommodates an annular phase ring of optimum size.

4.3.5 Limitations of Phase Contrast Microscope

- The images generated by a phase contrast microscope mostly have radiance in the vicinity of detail outlines that manifest a high phase shift. These radiance interferences are optical artifacts complicating the visualization of sample boundaries.
- Many times, a reduced resolution of phase images is obtained owing to the phase annulus restriction of the system numerical aperture.
- Intended modifications (condensers and objective lenses) contribute to significant expenditure in the microscope cost. As a result, phase contrast is often not used in teaching labs except exclusively for health professions.

4.3.6 Applications of Phase Contrast Microscope

Utility modules of phase contrast microscopy are numerous, wherein high-contrast and high-resolution images of the transparent specimen are the most significant. Some prominent biological samples and material patterns imaged distinctly and more informatively using phase contrast microscopy include:

- Living cells (often in a culture environment).
- Microorganisms, thin tissue slices, lithographic patterns, fibers, and latex dispersions.

- Subcellular structures (nuclei and other cell organelles).
- Provides a clear illustration of living cells' proliferation through distinct cell cycle phases. One can view the cytoskeleton dynamics during cell division, phagocytosis, etc.
- Allows the kinetic study of cellular events such as cell division and phagocytosis.
- Enables monitoring of membrane permeability of cells and different organelles.

The technique is extensively used to view living cells in tissue culture via monitoring their growth (McNamara 2005; Liu et al. 2014).

4.4 Comparative Analysis of Phase Contrast and Bright-Field Microscopy

Figure 9 depicts the images of HeLa cells captured using bright-field and phase contrast microscopy. It can be seen clearly that while phase contrast microscopy stains the cells darkly and provides a much clearer view, the visualization in a bright-field microscope is comparatively unclear.

- The typical appearance of a bright-field microscopy image is a hazy sample on a bright background and hence it is named so. The phase contrast mode confers contrast to cell membranes and boundaries, making the visualization easier and helpful for cell counting in densely cultured plates (Fig. 9).

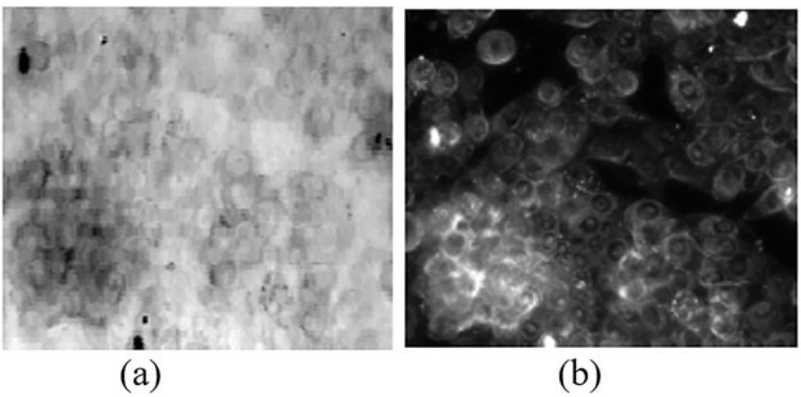


Fig. 9 Imaging of HeLa cells viewed using (**a**) bright-field and (**b**) dark-field modes, in a 20× numerical aperture, 0.4 objective lenses. The unstained biological samples exhibit low contrast in bright-field mode owing to inadequate natural pigmentation. Dark-field visualization enables contrast at cell membranes or periphery, which aids in cell counting in the culture plates (Liu et al. 2014)

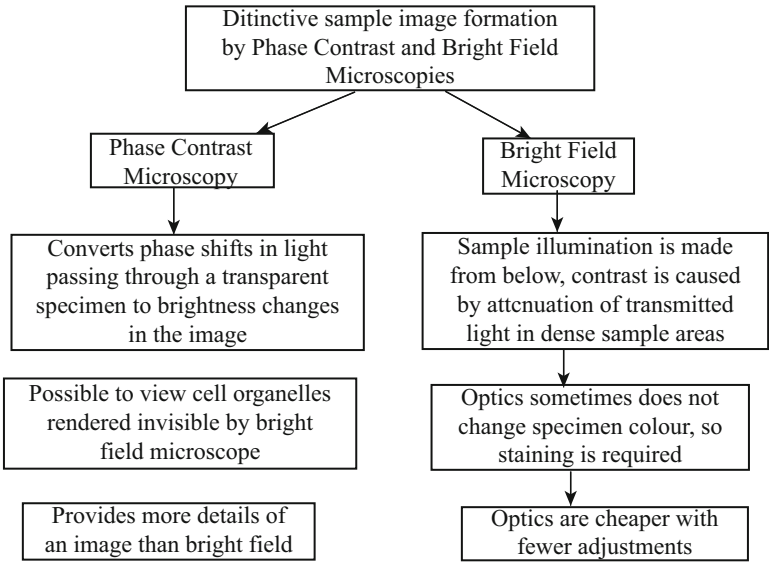


Fig. 10 Fundamental distinctions of phase contrast and bright-field microscopy

Figure 10 depicts the principle and working distinctions of a phase contrast and bright-field microscope, with the former being more suited to view the biological specimen due to distinct identification of the specimen.

For knowing about cell morphology, wherein knowledge of cell shape or volume is needed, phase contrast microscopy is the most suitable technique. For instance, some epigenetic modulators like Trichostatin A can change the epigenetic state of chromosomes that can change the cell morphology from round to elongated regime (McNamara 2005; Liu et al. 2014).

4.5 Confocal Microscope

- Unlike microscopic techniques (well suited for thin and optically transparent cultures), the imaging of three-dimensional (3D) cultures requires probes for imaging the thicker biological structures at high resolution. The major reasons behind this are thickness and high scattering of 3D cultures, forbidding light penetration without considerable distortion.
- Typically, three major techniques equipped with requisite modalities in this regard are *confocal microscopy (CM), multiphoton microscopy, and optical coherence tomography*. The working modules are equipped with probes for the safer assessment of cellular dynamics in 3D microenvironments repeatedly over varying time gaps.
- Approaches like “bright-field” and “phase microscopy” (frequently used to visualize live cells in 2D cell cultures) rely on transmitted light through the sample.

The nonfeasibility of these approaches to view cells in 3D cultures arises from their greater thickness (of sample view) which hinders effective light passage. So, for nondestructive imaging of 3D cultures and thick tissues, techniques capable of light collection in the backward direction are needed. These techniques (better referred to as **epi-illumination imaging methods**) work via ascertaining fluorescence and backscattered light. The specific focus is on visualizing the fluorescent marker intended to target a specific area or molecule of interest Pawley 1995; Paddock 1999; Müller 2002.

4.5.1 Invention of Confocal Microscope

- The scientific inspiration for CM development was provided by *Marvin Minsky*, in his quest to image the neural networks in unstained brain tissues, driven by the enthusiasm to image biological events in their natural state.
- While the unavailability of intense light sources and computer horsepower for imaging and handling large data led to Minsky's observation remaining unnoticed, ***Egger and Petran*** in the late 1960s fabricated a multiple beam confocal microscope (following Minsky's work), using a spinning disk to examine unstained brain sections and ganglion cells.
- Continuing in the domain, Egger was the first to develop the first mechanically scanned confocal microscope and went on to publish recognizable cell images in 1973.
- The present advancements of distinctive identification were manifested by the rigorous progression of computational processing and laser technology during the late 1970s and 1980s, enabling the application of precise algorithms for the digital manipulation of images.

4.5.2 Working Principle of Confocal Microscope

- The fundamental essence of CM lies in the fluorescence, noted as a consequence of sample illumination with a characteristic light color that results in the emission of a different colored light (lower energy than that of the incident). The typical genesis of the whole phenomenon is the absorption of a high-energy light by the molecules, resulting in their excitation to a higher energy state. Since the stability of the excited state is transient, the molecule concurrently reverts to the ground state losing proportionate quanta of energy (this energy is never similar to absorbed energy because of unpreventable energy loss during the transformation).
- The terminology "confocal" implies the rationale of twin lens assembly with spatial placements for similar focal points. Two main variants of the confocal microscope are **laser scanning microscopes** and **tandem scanning microscopes**, with the former suitable for **immunofluorescence** while the latter finds appropriateness for **high-speed reflection imaging**.
- A common dye used to confer fluorescent activity is **fluorescein**, emitting green light on being excited with blue light.
- The characteristic color of the emitted light depends on the nature of the material, the duration of exposure to the specific light source, the temperature of the source, and the operational conditions of the exposure arena.

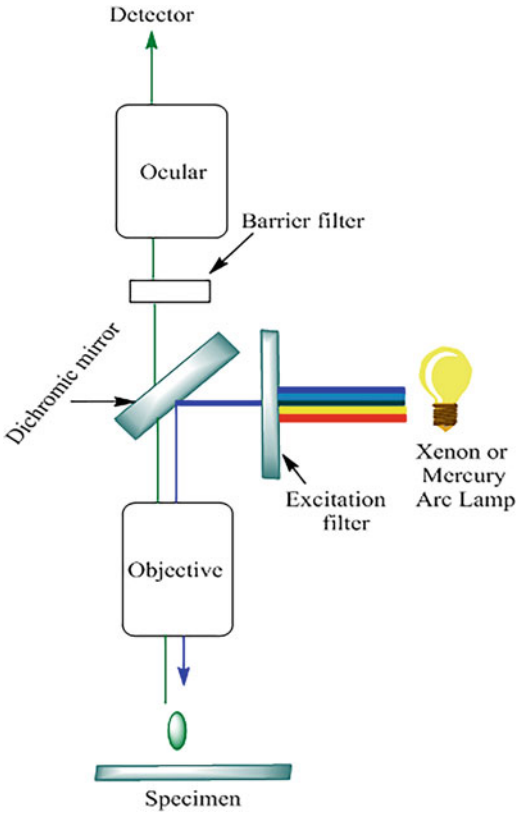


Fig. 11 Working configuration of a fluorescence microscope. Noteworthy distinctions from conventional microscopy include the elimination of out-of-focus light rays and enhanced sensitivity provided by laser scanning confocal microscope optical configuration

Figure 11 depicts the working configuration of a confocal principle in epifluorescence laser scanning microscopy. The coherent light incident by the laser system (excitation source) moves across the pinhole aperture situated in a conjugate plane (confocal) with the intended scanning location and a second pinhole aperture located in front of the detector (a photomultiplier tube). With progressive laser reflection by a dichromatic mirror and subsequent scanning across the analyzed sample in a designated focal plane, reciprocated secondary fluorescence emitted from distinct sample regions (in the same focal plane) passes back through the dichromatic mirror, before being subsequently focused as a confocal point(s) at the detector pinhole aperture.

- A substantial proportion of fluorescence emission at regions above and below the objective focal plane is not confocal with the pinhole. It is because these out-of-focus light rays form the extended airy disks along the aperture plane.
- Since only a small fraction of out-of-focus fluorescence emission is captured through a pinhole aperture, the majority of this light remains undetected by the photomultiplier, thereby no longer contributing to the resulting image.

- The excitation and emission points on the sample could be shifted to a new plane via refocusing the objective so that sample excitation and emission peripheries now prevail in a new plane that is confocal with the light source and detector pinhole apertures.

4.5.3 Key Components of a Confocal Microscope

- A typical CM assembly comprises **two pinholes** for a mandatory light passage and subsequent entry into the eyes of an observer.
- A **laser** is used to provide exciting light or light with very high intensities. The light as simulated emission of radiations first passes through the first pinhole before reaching the condenser lens.
- The **condenser** focuses this light on the specimen.
- After screening the specimen, light passes through the **objective (lens)** before being focused via second pinhole B.
- Typical performance determining subunits of a CM includes multiple laser excitation sources, a scan head comprising optical and electronic components, electronic detectors (usual photomultipliers), and a computer for recording, handing out, and displaying images.
- The scanning head is the central feature of the entire system, exclusively responsible for rasterizing the excitation scans besides retrieving the signals from the specimen for assembling the final image. This unit gathers inputs from the external laser sources, fluorescence filter sets and dichromatic mirrors, a galvanometer-based raster scanning mirror system, variable pinhole apertures for generating the confocal image, and photomultiplier tube detectors tuned for different fluorescent wavelengths.
- An important requirement of integrated CM working is the interconnectivity of the excitation laser beam and the scanning unit with a fiber optic coupler followed by a beam expander. This beam expander enables a thin laser beam wrist to fill the objective rear aperture. It is the scanning unit that houses internal fluorescence filter components (i.e., excitation and barrier filters along with the dichromatic mirrors). This unit also regulates the control operations of interference and neutral density filters, through rotational provisions of sliders to strengthen the coherence of incident light beam.
- Pinhole aperture forms an important component of the scanning unit, acting as a spatial filter at the conjugate image plane positioned immediately in front of the **photomultiplier.**
- External regulations of the pinhole turret allow for adjusting the pinhole size, thereby controlling the amount of light entering inside.
- The aperture excludes the fluorescence signals from out-of-focus regions positioned above and below the focal plane, which are subsequently projected to the aperture as **airy disks**. These airy disks have a significantly larger diameter than the ones actively involved in forming an image and are spread over a larger area owing to which only a small fraction of light originating in planes away from focal points passes through the aperture.

- Pinhole aperture also regulates the amount of incident light by eliminating a substantial proportion of stray light passing through the optical system.

4.5.4 Distinction from Widefield Microscopy

- A comparative assessment of similarities and distinctions in the wide field and confocal microscopic analysis provides a better understanding of the nature and geometry of specimen illumination in each of the methods. Wide-field epifluorescence microscope objectives focus a wide cone of illumination over a large sample volume, paving way for uniform and simultaneous illumination. Most of the fluorescence emission is reverted to the microscope that is subsequently gathered by the objective (depending upon numerical aperture, NA) for projection onto the eyepieces (or detectors). The net result of such wider sample scanning is the impaired resolution and image contrast due to significant signaling formed by emitted background light and autofluorescence arising from regions above and below the focal plane.
- In CM, the laser illumination source is first expanded to occupy the objective rear aperture followed by focusing through the lens system to a very small spot at the focal plane.
- The size of the illumination point is generally within 0.25–8 μm in diameter (depending on the objective NA) with 0.5–1.5 μm depth corresponding to the brightest intensity.
- Spot size determination is affected by **microscope design, incident laser light wavelength, objective characteristics, scanning unit settings, and the specimen**.
- **Figure 12 contrasts** the illumination cones of a wide-field and point scanning confocal microscope having the same NA. As opposed to wider area illumination in a wide-field microscope, a confocal microscope scans a finely focused spot on the focal plane.

4.5.5 Analysis with a Confocal Microscope

- There are some lenses inside the microscope, which focus light from the focal point of one lens to another point (located on the sample being analyzed).

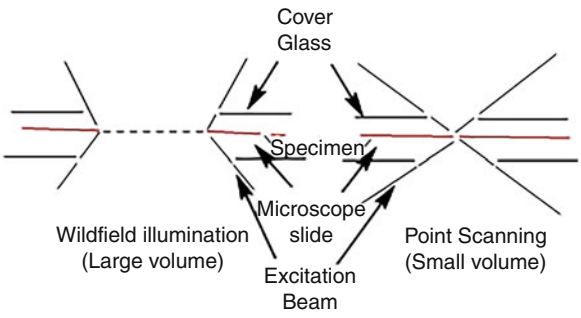


Fig. 12 The sample imaging distinctions in wide-field (electron microscopy) and point scanning (CM) modes

- The sole motto is to obtain the image of a point lying directly on the focus.
- To achieve this, a screen having a pinhole is placed on the side opposite where the object is placed. This arrangement enables the entire incident light to pass through the pinhole.
- At the same time, most of the light from the second focal point is out of focus on this screen and often gets blocked by the pinhole.
- CM enables the high-resolution optical sectioning of thick samples, with a typical penetration length being <100 μm.
- There are several variations of CM but the technique has gained significant interest in life sciences, in its most important version entitled "confocal laser scanning microscopy." This method of cell visualization is based on point illumination of a sample with a laser followed by spatial filtering of the returning beam with a pinhole to obstruct the light from outside the focus. The beam is gradually scanned across the sample, leading to the development of an image. The working mode could be fluorescence or reflectance, with the former being preferred for the tissues capable of distinctive identification concerning their chemically complex and heterogenic physiological vicinity.
- During analysis, the whole sample is illuminated by the exciting light owing to which the complete sample region undergoes fluorescence at the same observation instant. The highest intensity of exciting light is received by the sample region coinciding with the focal point of the lens, although other sample regions are also subjected to fluoresce to varying extents.
- Such distinctions in fluorescence abilities of sample regions result in a background haze in the image that is resolved by adding a pinhole to the overall assembly.
- The focal point of the objective lens is adjusted to form the image at the pinhole locus, making the pinhole conjugate to the focal point of the lens.
- Specified fluorophores are available for a distinguished view of mitochondria, chloroplasts, Golgi apparatus, and other cell organelles, corresponding to well-defined excitation and emission wavelengths.
- The present research on FM is actively focused to develop probes that could be excited by near-infrared wavelengths, to improve the penetration depth that remains currently restricted to <100 μm.
- The success of a fluorescence imaging system critically depends on the stability of probes, allowing minimum cross-reaction as well as nondegradable prevalence in the in vivo conditions.

4.5.6 Sample Scanning in Modern Confocal Microscopes

- The current preciseness and analysis accuracy of the confocal approach are the outcome of significant advances in **optical** and **electronics technology**.
- The advent of stable multiwavelength laser systems allows better coverage of ultraviolet, visible, and near-infrared spectral regions, better control of interference filters, precise low noise wideband detectors, and much more powerful computers.

- Current microscopic systems accomplish the sample scanning either by translating the stage in all three dimensions (while holding the laser illumination spot in a fixed position) or via faster scanning of the beam itself across the specimen.
- Since three-dimensional conversation on the stage is difficult and more susceptible to vibrational interferences, extensive instruments in the modern arena use a beam-type scanning mechanism. The beam scanning utilizes single and multiple beam scanning as two fundamental modes, with the former involving a pair of computer-controlled sample scanners working in a raster pattern at the rate of one frame per second while the latter works through *spinning Nipkow disk*, constituted of multiple pinhole arrays.
- Scanning rates could be fastened using oscillating mirrors.
- The major advantage of the multiple-beam scanning mode is the use of arc-discharge lamps for sample illumination, thereby reducing sample damage with much-improved detection of low fluorescence levels.

4.6 Factors Affecting Imaging Resolution

- Spatial resolution in CM depends on the NA of the objective, the pinhole size, and the wavelength of incident light.
- Resolution in the range of micrometers could be achieved using commercial systems allowing the visualization of even the tiniest cells and subcellular features.
- Penetration depth in thick samples is restricted by the fact that scattering often results in defocusing of the illuminating light beam. Consequently, this reduces the amount of light passing through the pinhole with progressively increasing cell depth. Ultimately, it limits the probe imaging depth.
- Other factors affecting penetration depth include optical properties of the specific cell type being analyzed, the NA of the objective, and the wavelength of a characteristic light source. In general, the light of a longer wavelength penetrates deeper within the tissue owing to its presumable low absorption and scattering.
- Fluorescence CM extensively utilizes shorter wavelength (near to visible) light radiations to excite commercial fluorophores via simulated absorption.
- Contrary to this, reflectance CM does not pose any strict restrictions on incident light wavelengths, thereby offering feasibility to improve penetration depth.

4.7 Some Specific Illustrations

Commonly, the CM specimen analyzing optical sections is not restricted to the lateral (x-y) plane and could be retrieved or portrayed in transverse planes.

Vertical sections in the x-z and y-z planes (parallel to the microscope optical axis) can be readily generated using most confocal software programs. Thus, the specimen seems as if being sectioned in a right-angle plane concerning the lateral axis. Usually, the vertical sections are obtained through a series of x-y scans taken

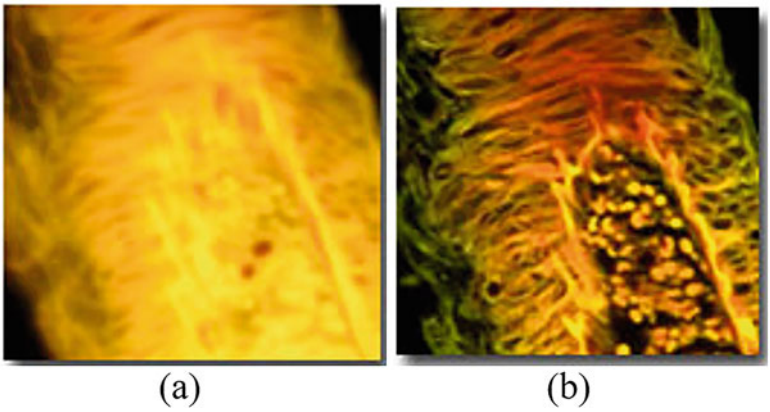


Fig. 13 Comparative images of fluorescence-stained human medulla using (**a**) traditional wide-field fluorescence and (**b**) laser scanning confocal microscope (retrieved from https://www.olympus-lifescience.com/en/microscope resource/primer/techniques/confocal/confocalintro/)

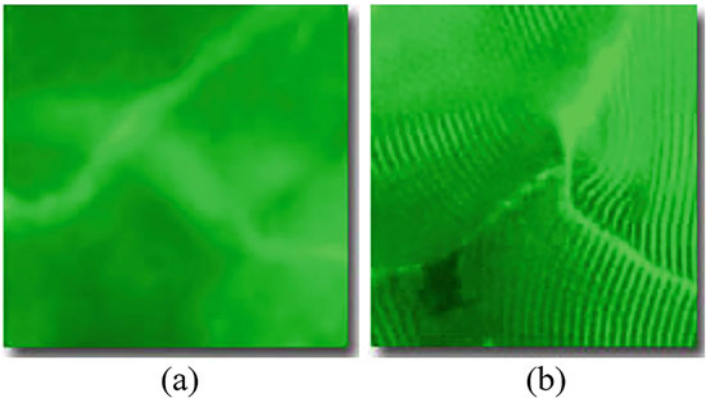


Fig. 14 Typical comparisons of whole rabbit muscle fiber topography analysis, viewed using (**a**) wide-field fluorescence and (**b**) laser scanning confocal microscope (retrieved from https://www.olympus-lifescience.com/en/microscope resource/primer/techniques/confocal/confocalintro/)

along the z-axis after projecting the fluorescence intensity as it would appear in the case of microscope hardware capability to perform a vertical section.

Figure 13 compares the fluorescent stained human medulla images using wide-field (traditional) and laser scanning confocal fluorescence microscopy. It is quite evident that wide-field fluorescence results in a persistent glare from the fluorescent structures above and below the focal plane (Fig. 13a). On the other hand, the laser scanning confocal microscope reveals considerable details of the structural morphology.

Figure 14 depicts the fluorescence images of whole rabbit muscles stained using wide-field fluorescence and laser scanning confocal microscopy, from which it is

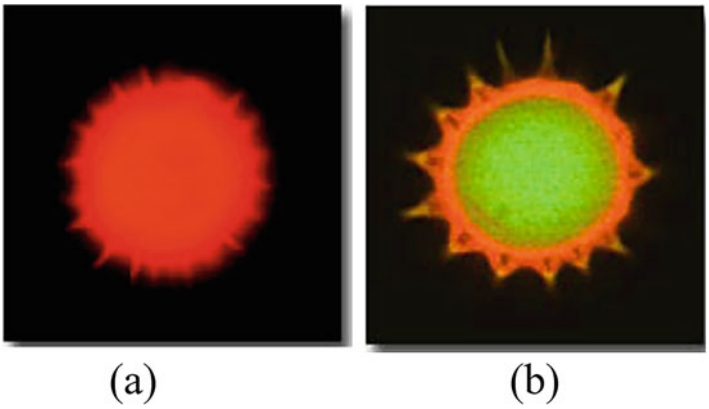


Fig. 15 Typical morphologies of the sunflower pollen grain, captured via (**a**) wide-field fluorescence and (**b**) laser scanning confocal microscope (retrieved from https://www.olympus-lifescience.com/en/microscope resource/primer/techniques/confocal/confocalintro/)

quite clear that traditional wide-field fluorescence produces significant blurring owing to which no information about the internal structural organization is inferred. On the other hand, the laser scanning confocal microscopy gives a much clear image revealing a consistent and striated topography of constituent muscles. These comparisons elucidate a better imaging resolution of laser scanning confocal microscopy, manifested through the laser technology in narrowing the wavelength extremes of the incident light, consequently producing the image out of similar phase maximum intensity of incident light waves.

Another comparison reveals a missing distinction of the outer periphery in a sunflower pollen grain with no indication of internal structure, using wide-field microscopy. Contrary to this, the same grain sample when viewed as a thin section using confocal microscopy reveals significant differences between the particle core and surrounding envelope (Fig. 15).

Provisions in computational software have enabled the analysis of much more complex specimens compared to pollen grains, having interconnected structural elements that are much more difficult to be retrieved through successive optical sections across the sample volume. However, using volume-rendering computational techniques, adequate series of optical sections could be gathered enabling easier processing into three-dimensional presentations. This approach is presently gaining increased scientific favor, bettering the structure-function understanding of cells and tissues through concurrent biological investigations. The necessity of adequate data collection mandates a recording of optical sections at appropriate axial intervals, through which actual specimen depth is reflected in the image. The structural makeup of a laser scanning confocal microscope resembles a familiar inverted research-level optical microscope, replacing the tungsten-halogen or mercury-based arc-discharge lamp with one or more laser systems (as a light source) that excite fluorophores in the specimen. Image information is retrieved through

point-by-point analysis using a specific detector (either via a photomultiplier tube or an avalanche photodiode), followed by processed digitization by the host computer. The collection and display of images are regulated by the provisions in the host computer, on which analysis can be made after a series of images have been acquired and stored on digital media (Heimstädt 1911).

4.8 Key Distinctions from Conventional Microscopy

- CM promises better control of field depth and background elimination information away from the focal plane (which could result in image degradation).
- CM has a higher ability to isolate serial optical sections from thick specimens.
- Provision of spatial filtering to eliminate out-of-focus light, having a thickness higher than the immediate plane of focus.
- CM does not require any specific sample preparation, benefiting in terms of obtaining high-quality images from the specimens optimized for conventional fluorescence microscopy.
- The key distinction between conventional and CM, in terms of optical features, is the presence of confocal pinholes in the latter, allowing only light from the plane of focus to reach the detector.
- CM produces images with nearly 1.4 times more impressive resolutions than conventional microscopy. This is possible because spatial filtering enabled out-of-focus light elimination. This provision helps to eliminate flare in samples having a thickness greater than the plane of focus. Computer software can be used to digitally reconstruct 3D sample illustrations, with ~0.5 and 0.2 μm vertical and horizontal resolutions, respectively.
- Unlike electron microscopy, CM does not result in any disturbance of native cellular physiology, with each organelle being scanned through a specific fluorescent dye. This enables the highest contrast and minimum attenuation which may be generated through an instantaneous cross-reactive response (Pawley 2006).

5 Cell Counters

Counting the number of cells (**live/dead cells**) present in a cell culture medium or other counting solutions is highly essential for successful mammalian cell culture technology.

***This has the following utilities*:**

- Aids in splitting or passage fractionation of cells
- Aids in transfection of mammalian cells.
- Simplifies the downstream processing of a large number of cells.
- Helps to ascertain the concentration of drugs/toxins/chemicals/regents necessary to check their effectiveness in controlling cell cycle/viability/or apoptosis.

- Finally, cell counting is decisive for all cell and cell-related research, including molecular biology, biochemistry, and genetics.
- Throughout life sciences and research, all investigations on cells analyze the results based on the number of cells present.
- Finally, cell kinetics, a fundamental discipline of cell biology for investigating normal tissue biology and diseases like cancer, requires cell counting to study differences in cell survival, proliferation, development, and aging.

***At present two methods are used for mammalian cell counting**:*

1. A manual method of cell counting
2. An automated cell counter

- While the manual method (using a **hemocytometer)** is the original method of cell counting and is used for more than 100 years (Absher 1973; Rouge 2002), the automated method is the most up-to-date counting method, based on the principle of some sophisticated technology such as *image-based technology, impedance counting technology, and flow cytometry.*
- Although automated cell counting methods can be preferentially chosen by any researcher because of significant advantages over the manual method, the hemocytometer is still in use in large parts of the world since it is not only cheap and affordable but can also be easily followed by anyone.
- The following paragraphs first describe manual cell counting by hemocytometer and then automated cell counting method.

5.1 A Manual Method of Cell Counting.

- For over 100 years, the hemocytometer is being used by cell biologists to count cells.
- It was invented by *Louis-Charles Malassez* for quantifying the blood cells, but with time it became a popular and effective tool for counting a variety of other cells including various mammalian cells.
- Currently, a hemocytometer is equipped with improved *Neubauer grids*.
- Hemocytometer is a microscope slide-like small device.
- This thick glass microscope slide with rectangular indentation creates an **H-shaped chamber** in the middle. This H-shaped chamber is carefully crafted and engraved with a laser-etched grid of perpendicular lines so that both the areas bound by the lines and the chamber depth are known. The H-shaped groove in the center has two chambers, further divided into nine (1.0mm $\times$ 1.0 mm) large squares, separated from one another by triple lines.
- The area of each large square is 1 mm^2, thereby making it possible to count the number of cells or particles present in a given volume of suspension.
- Cells are counted by determining their number per ml of suspension.

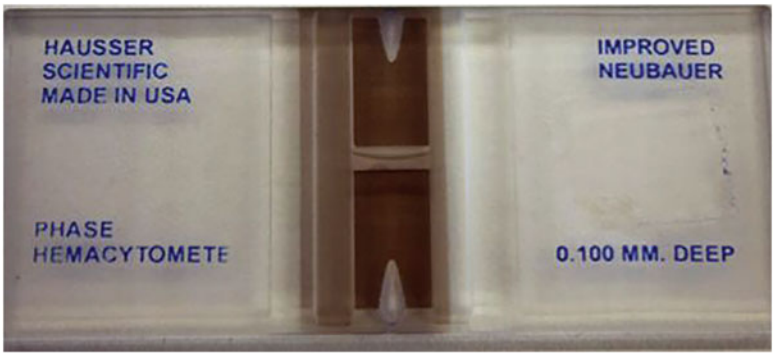
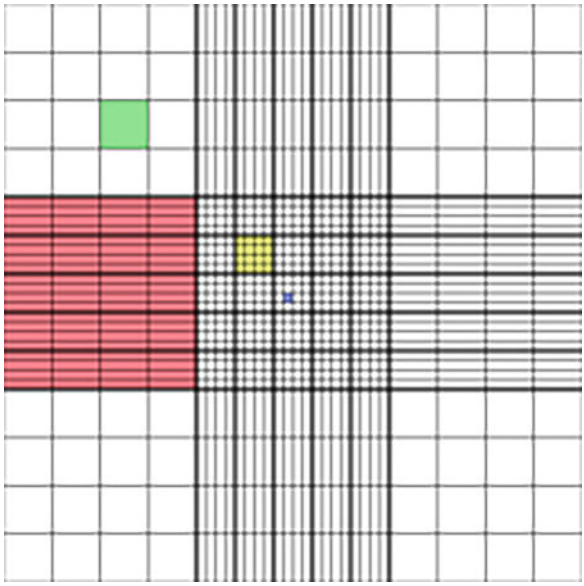


Fig. 16 Hemocytometer with H-shaped chamber in the middle

Fig. 17 Hemocytometer grid (see also table)



- The cover slips used in hemocytometer are specially manufactured, thicker and heavier than the regular 0.15 mm thin cover slips, used for clinical purposes. Hemocytometer cover slips are heavy enough to overcome the surface tension of the liquid drop. This special cover slip is always placed on the hemocytometer before putting it on the cell suspension (Fig. 16).

5.2 Description of Various Hemocytometer Chambers and Their Use

- Structurally, the hemocytometer grided area consists of several 1 mm × 1 mm (1 mm^2) squares. Further, these squares are subdivided in three directions, namely, 0.25 mm × 0.25 mm (0.0625 mm^2), 0.25 mm × 0.20 mm (0.05 mm^2), and 0.20 mm × 0.20 mm (0.04 mm^2) (Fig. 17).
- As described in the hemocytometer manual, the central square is further subdivided into 0.05 mm × 0.05 mm (0.0025 mm^2) squares. The area of each corner and middle square is 1 mm × 1 mm = 1 mm^2.
- Finally, the depth of each square is 0.1 mm which retains 100 nl volume.
- Before putting the cell suspension in the V-shaped groove inside the gridded chamber, the thicker (than usual) hemocytometer cover slip is placed on the grid. As the glass cover slip is supported over the chambers above 0.1 mm height, the entire counting grid lies under 0.9 mm^3 volume on one side.
- As the cover slip is put on the grove, it is ensured that the two glass surfaces are in proper contact with each other via *observing Newton's ring*.
- Upon adding the coverslip, the cell suspension is loaded in the V-shaped groove. The capillary action generated in between grove and cover slips distributes the cells into the void area that fills the chambers.
- Now the cells within the chambers (not in the borderlines) are counted manually using a microscope (either phase contrast or bright field).

5.3 Counting Cells Using Hemocytometer

5.3.1 Materials and Instruments for Cell Counting Using Hemocytometer

1. Uniform cell suspension.
2. Cell culture tubes for keeping cell suspension.
3. Trypan blue.
4. Phosphate-buffered saline (**PBS**).
5. Hemocytometer/cell counter.
6. Hemocytometer cover slips.
7. Inverted microscope (**preferably phase contrast**).
8. Micropipettes and tips.

5.3.2 Method of Cell Counting Using Hemocytometer

1. Prepare a 1:100 dilution of uniform cell suspension in a phosphate-buffered saline by mixing 10 µl original cell suspension and 990 µl PBS gently via pipetting up and down using a micropipette.
2. Clean the hemocytometer and its cover slip carefully by wiping with 70% ethanol and making it grease-free.

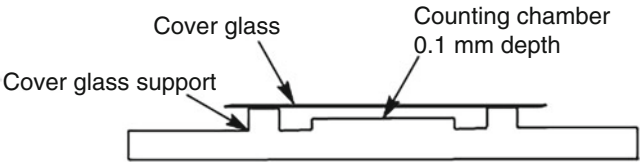


Fig. 18 Components of a hemocytometer, side-view coverage

3. Put the cover slip on the hemocytometer after loading the diluted cell suspension into the "V" groove so that the cell suspension is drawn into the designed space via capillary action.
4. Place the hemocytometer under the microscope stage and adjust focus at low power to see the counting grid (Fig. 18).
5. Observe the cells at 40× magnification and count manually or using a cell counter.
6. **Conventional rules for counting:** For counting large cells (e.g., WBCs), the four large corner squares are used, whereas for small cells (e.g., RBCs), the central small squares are considered.

   NB: Specific instruction is given in the hemocytometer manual regarding which cells are to be counted. The manual further states the appropriate instruction for accurate counting of cells using a hemocytometer.

   The instruction is as follows:

   > Cells touching the left and top lines are counted while those touching the bottom lines and the right lines should not be counted.

7. Determine cell concentration using the following formula after the cell counting:

   Cell concentration in a cell suspension = Total cells counted in large square × dilution factor × 10^4 cells/ml

   The statistical error in cell counting using a hemocytometer should be between **10% and 15%.**

   NB: The addition of trypan blue is not necessary if cell counting is the only purpose. However, if the purpose is to separately count live and dead cells, the addition of trypan blue in the cell suspension is necessary since it exclusively stained plasma membrane deformed and dead cells.

5.4 Important Points to Be Considered for a Perfect Cell Count

- The original cell suspension must be uniform for representative and correct cell counting. So, before cell count, mix the cell suspension well by pipetting up and down repeatedly. A nonuniform suspension leads to an erroneous cell count.
- If a cell suspension is too concentrated, it may lead to erroneous and nonuniform counting. Hence, the sample should be serially diluted with saline or **PBS**. The dilution of cells **should never be made with water** as it will cause **osmotic shock**

and subsequent cell burst. So, either cell culture medium or preferably PBS (isotonic to cells) may be used for dilution.

- Use hemocytometer cover slip only rather than regular cover slips as these are less flexible and heavy enough to overcome the surface tension of liquid drop.
- Remove the bubbles from the hemocytometer after loading cells before counting.
- The chamber should not be overfilled or under-filled. Improper filling may lead to errors in cell count.
- *The use of paper wipes is not advised to dry the excess liquid.* The counting chamber will then dry it out and produce an erroneous cell count.
- Moving cells, like sperm cells, are difficult to count. Such motile samples should be first immobilized using methanol as a dilution agent instead of a regular buffer.
- It is advisable not to crash the microscope objective into the hemocytometer, during focusing.
- It is also advisable to perform a duplicate count of the same cell sample to get the average cell count. If the results are too different, either the original cell suspension is not uniformly mixed or dilution is not correctly done.

  NB: Protective clothing, gloves, and eyewear should be always used during cell counting with trypan blue as it is a mutagen. Clean the hemocytometer as soon as possible before the cells are dried. Immediately after use, the hemocytometer and its cover slip are rinsed well with distilled water to remove trypan blue before wiping with 70% alcohol and allowing it to dry.

5.5 Counting Viable Cells Using Hemocytometer

Several stains like trypan blue, erythrosine B, and nigrosine are used to identify viable and nonviable cells. All viable cells exclude stains though the membrane looks bright under a microscope. Cells not taking any stain are considered viable. Once the stain enters the dead or damaged cells, it is not excluded and appears colored under a microscope. Thus, total cells are a sum of colored and uncolored bright cells. A cell suspension is considered dead if >20% of total cells remain stained.

5.6 Common Use of Hemocytometer

5.6.1 Hemocytometer Is Used for Cell Counting Concerning the Following Parameters

Cell Culture

To view and count cells during subculture or monitor cell proliferation of a growing culture at various time intervals

Cell Viability

Estimating viable cells by counting brightly stained cells

Cell Size

The real cell size can be inferred by scaling it to the width of a hemocytometer square, estimated by fitting the scale in a micrograph.

Cell Processing in Biotechnology for Downstream Analysis

Accurate cell numbers are needed in many cell-based assays, particularly flow cytometry.

Pathological Blood Counts

For manual cell counting, where automated cell counters do not work well (in patients having abnormal blood cells)

Sperm Counts

Sperm cells are motile and, thus, can be counted using a hemocytometer, however, after immobilization.

Fermentation Industry

Hemocytometer is used for yeast preparation during beer brewing.

5.6.2 Limitations of the Hemocytometer

Several shortcomings are described in cell counting using a hemocytometer. It is a manual technique and therefore tedious and time-consuming. Additionally, the negative points include but are not limited to statistical robustness at low sample concentration, poor counts due to device misuse, and subjectivity of counts among the users. To overcome at least some of these limitations, automated cell counter could be used (Marlene 1973; Nielson et al. 1991; Davis 1995; Rouge 2002).

5.7 An Automated Method of Cell Counting

As described in the title following sample (cell suspension) loading, an automated cell counter counts the cells robotically. As a part of the procedure, the cells are forced through a small tube on sample loading, while the counter uses **optical or electrical impedance sensors** to count the cell number (Shah et al. 2006; Sireci et al. 2010).

The automated cell counting can be divided into the following three major categories:

1. Image-based technologies
2. Impedance counting technologies
3. Flow cytometry-based methods

Here is a brief discussion of the above techniques:

5.7.1 Technologies Based on Cell Images

These methods rely on the principle of automatic cell counters wherein images of every cell are generated (captured) and are subsequently counted. To make the cell images, the software must recognize the cell size. Specific instructions are given by the vendors such as *Invitrogen* and *Nexcelom*

The *basic procedure includes the following steps*:

- Set the microscope for viewing the cells.
- Capture the images of the cells.
- Identify the cells based on their **diameter**.
- After a direct count of the cells, the dead cells are stained **using the trypan blue exclusion technique (the dead cells' cytoplasm appears in blue, whereas live cells do not exhibit any color)**.
- Now the dead cells are counted to know the exact cell number.

  NB: Negative point of image-based technology. This procedure requires a specialized slide or cassette, which adds to the cost.

5.7.2 Cell Counting Technologies Using Impedance

- In 1953, it was *Mack Fulwyler* who developed the first cell sorter. It was the **coulter principle** that formed the basis of modern complete blood counts (**CBC**).
- In the **Coulter counter, a probe comprising of two electrodes separated using a small hole inserted into a solution containing the particles to be counted on the cell surface.**
- The **impedance** (effective resistance of an electric circuit or component to alternating current) between the two electrodes is disrupted as the particles pass through the hole.
- The particle size distribution determines the impedance which is **correlated to mobility, surface charge, and concentration of the particles.**

Limitations of Counting Technologies Using Impedance.

Some limitations of this technology include the followings:

- A possible clogging of the office if the concentration of cells is too high.
- Dead cells are identified based on size and impedance signal changes.
- The impedance measurement cannot be possible if the cells are not diluted in a proper concentration with a certain electrolyte concentration.
- **Examples:** Beckman Coulter and EMD Millipore. The Millipore instrument is sometimes also referred to as the Scepter. It is a handheld device that is like a pipette besides facilitating a rapid sample count, multiple times.

5.7.3 Flow Cytometry-Based Technologies

- A flow cytometer (also called cell sorter) is the most sophisticated instrument used to ascertain the **number, size, and nucleic acid content of cells moving in a narrow stream through a beam of light (laser beam).**

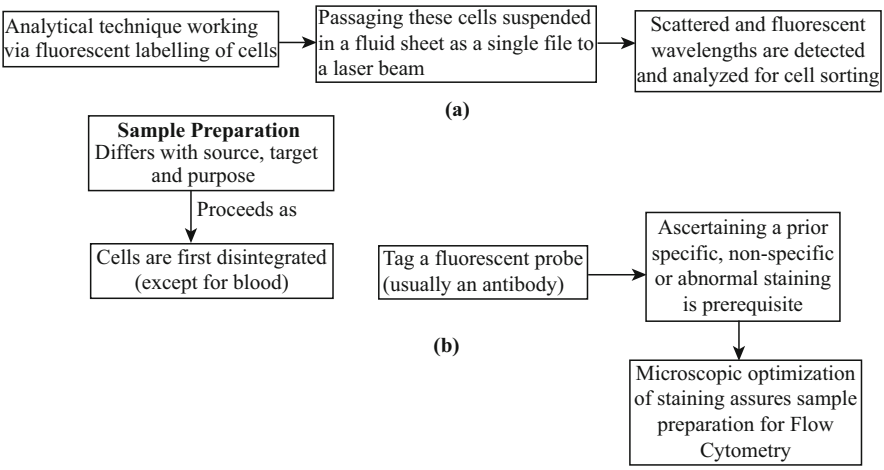


Fig. 19 Working principle (**a**) and sample preparation conditioning (**b**) in a flow cytometer. Sample preparation mandates a prior screening of residual staining impact

- Additionally, this instrument is used to analyze the **cell** shape along with its **internal and external structures**.
- Finally, the flow cytometer is also used for measuring the number of **specific proteins** and **other biochemicals**.
- These proteins help to **sort the cells** based on their cell cycle **(G1, S, G2, and M phase)** involvement.
- Procedure-wise, the laser beam hits the cells one by one, after which a light detector gathers the reflected light (Fig. 19).
- **NB:** As indicated above, a flow cytometer is also called a **cell sorter**.
- While the instrument is costly, it has several applications as indicated above. Therefore, laboratories may purchase this instrument in addition to other techniques for cell counting.

Syringe/Pump Systems

Certain companies such as **Chemyx** developed these pumping devices. According to the company manual, this kind of pump system provides a reliable operation for the development of impedance-based techniques for cell counting and identification. The microfluidic operation is required for this system (for a large number of cells, flow cytometry is the best option). In this technology, it is possible to miniaturize and integrate with impedance tools.

NB: For further studies, the company webpage can be checked.

Differential Pressure Systems

- For the cytometers using differential pressure systems (e.g., LSR-II or Ganferllios), the transferred volume is not accurately measured; hence, a proportional counting bead can be used to maintain the accuracy.

- Such beads are available at *BD Bioscience, Life Technologies, and Spherotech* among others.
- To use these beads, a sample is spiked with a defined volume of the beads. These are measured separately using the cells of interest, after which the number of countable beads per sample is determined to know about the correct number of cells. Of course, these beads will add to the experimental cost. Cell counting is a critical task for good flow cytometry.
- There are multiple ways to determine the cell number in a sample, each one having its implicit strengths and weakness.
- From the gold standard of the hemocytometer to the automated technologies, there are various ways to ascertain the cell number in an examined sample.

5.7.4 Usefulness of Cell Counting Made Through Automated Instruments

Here are some applications of automated cell counting instruments:

- Faster than the manual hemocytometer operation.
- A relatively large number of cells can be counted contrary to the manual technique.
- Automated cell counting is more accurate than manual techniques since there is no bias.
- The exact ratio of live-dead cells.
- Easier statistical calculation(s).

6 Cell Culture Containers, Transfer Aids, Membrane Filters, and Glasswares

Here is a brief discussion about the cell culture containers, transfer aids, membrane filters, plasticware, and glassware.

6.1 Cell Culture Containers

- The choice of the plastic culture vessels depends on the nature of the experiments, the number of cells required to be cultured, and, of course, the cost of the plasticware.
- Cell culture flasks, plates, and Petri dishes of various sizes are used as cell culture vessels.
- In general, the flask sizes that are most commonly used are 25, 75, and 150 mm. Similarly, Petri dishes having the sizes of 30, 60, and 100 mm and plates having 96, 48, 24, 12, and 6 wells are widely used (Fig. 20 depicts the various cell culture containers).
- Most mammalian cells (e.g., endothelial cells) are adherent in nature. Few blood cells, e.g., B/T cells, are cultured in suspension.

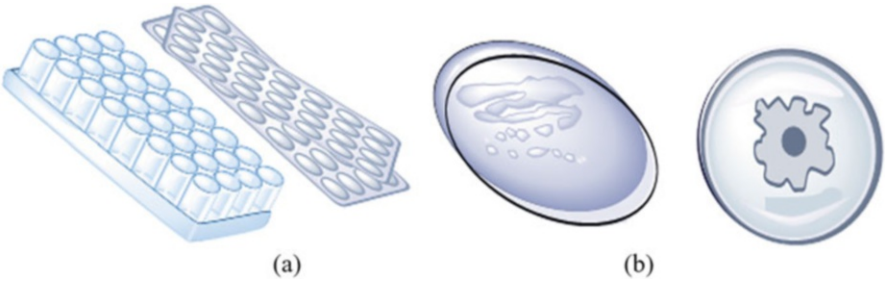


Fig. 20 Pictorial views of (**a**) culture plates and (**b**) Petri plates of different capacities

- For adherent cells, *disposable polystyrene-made* Petri plate/flasks are used for the culture.
- For strictly adherent cells (e.g., endothelial cells), the polystyrene-made cell culture containers (the bottom surface where the cells will grow) are further treated with **gelatin, laminin, poly-L-lysine, fibronectins,** etc., enabling their further attachment.
- Since the abovementioned molecules are the constituents of the extracellular matrix **(ECM**), adherent cells grow very well on these surfaces.
- The hydrophobic surface of *virgin polystyrene* culture vessels is converted to hydrophilic, using special treatment during manufacturing to allow the attachment of anchorage-dependent cells.
- The anchorage-dependent cells often form a **monolayer (except for cancer cells)** and cover the surface available to them.
- Flat-bottomed plasticware is naturally used for this purpose to ensure an increased surface for cell growth.
- In contrast, anchorage-independent cell types do not require any anchorage to sustain their growth.
- Virgin polystyrene is *hydrophobic* and is used to manufacture many cell culture flasks or plates.
- As cell adhesion molecules do not attach to this hydrophobic surface, virgin polystyrene tubes are suitable for culturing **anchorage-independent**, **non-adherent cells**.
- As a result, the plasticware made of virgin polystyrene is used to grow anchorage-independent nonadherent cells and is not treated for cell attachment.
- They can be grown in suspension cultures and their survival does not need any extracellular proteins at all.
- The anchorage-independent mammalian cells are limited in number, e.g., some hematopoietic cells.
- Sterile stirrer bottles are normally used for agitation of the aggregated cells in a culture so that cell clumps are broken and cells remain in suspension.
- Both anchorage-dependent and anchorage-independent cells are **aerobic**.
- Both cells in culture need O_2 and CO_2 exchanges for growth.

- The loose-fitting lids and the caps of the mammalian cell culture flasks must be closed loosely to allow gas passage.
- However, at present many companies have developed flasks having vented caps with an opening covered with a filter. This will allow contamination-free gaseous inflow inside the flasks (Balin et al. 1977; Gabridge 1985).

6.2 Transfer Aids

- For accessory materials of cell culture use of 15 ml and 50 ml disposable sterile **polypropylene** and **polycarbonate centrifuge tubes**, **sterile pipettes (serological and micropipettes)**, **sterile tips** for **micropipettes,** and **sterilization filters** are important (Fig. 21 shows centrifugation tubes and pipettes).
- 15 and 50 ml **plastic centrifuge tubes** are used for harvesting the mammalian cells. These are available either in sterile, disposable configurations or as reusable, autoclavable types.
- Disposable, sterile plastic serological pipettes of various sizes (1, 2, 5, 10, 25, and 50 ml) are used for the transfer of medium and other materials of varying volumes.
- Electronic automatic pipette aids are fitted with the serological pipettes to facilitate the easier transfer of liquids. Similarly, micropipettes of 0.2–2 µl, 2–20 µl, 20–200 µl, and 200–1000 µl capacities are preferred for the transfer of small volumes.
- Sterilized **plastic tips of different sizes** are fitted with these micropipettes to take and dispense liquids in a sterile manner.
- Both micropipettes and pipette aids are routinely disinfected by wiping with 70% alcohol before and during sterile work.

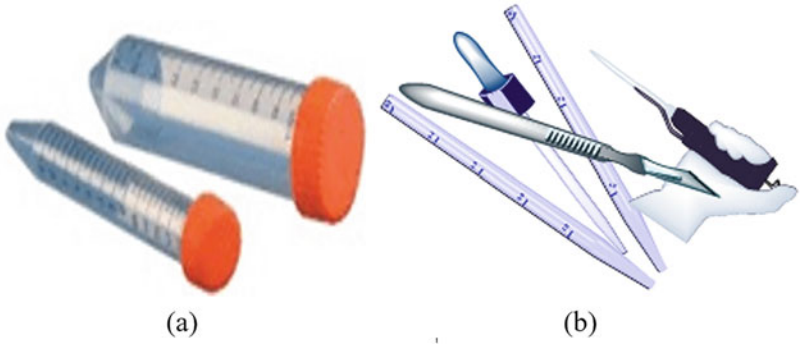


Fig. 21 Varying capacity (**a**) centrifuge tubes and (**b**) serological pipettes used for mammalian cell culture

6.3 Membrane Filters

- **Sterilization filters** are used to sterilize medium and other reagents that cannot be heat sterilized.
- Bottle-top or flask filters are available commercially.
- Disposable company filters of low protein binding capacity (**0.2 µm size)** are only suitable for this purpose.
- Filter apparatus are available with disposable **0.22 or 0.45 µm** membranes, having low protein binding capacity and capable of being sterilized by autoclaving.

6.4 Glasswares

- The major glassware used for mammalian cell culture includes medium storage bottles, conical flasks, microscopic slides with coverslips, Pasteur pipettes, and test tubes.
- All glassware is **dipped in acidic water overnight instead of detergent, enabling** thorough washing with excess water. Subsequently, these are either cotton plugged tightly or corked with loosely fitted corks and wrapped with aluminum foil properly to avoid contamination.
- Later, they are sterilized by moist heating (autoclaving) followed by dry heat using an oven.
- Only sterile and dry glassware is used for mammalian cell culture.

Fig. 22 Snapshots of (**a**) benchtop and (**b**) tabletop centrifuges

7 Centrifuges

A centrifuge is an instrument in which liquid samples containing suspended cells, cell lysate, or blood are spun at various speeds. In general, centrifuges work on three-dimensional principles, utilizing centripetal acceleration to separate particles based on higher and lower density (Mikkelsen and Eduardo 2004) (Fig. 22).

- In a cell culture laboratory, tabletop centrifuges are used to harvest cells from the culture medium.
- For cell harvesting, simple low-speed centrifugation at 1000–2000 revolutions per minute (**rpm**) and 4 °C is used. The use of low temperature (4 °C) for centrifugation is sometimes recommended to avoid possible cellular disruption at high temperatures.
- There are various types of centrifuges, depending on the size and sample capacity. Mostly, a tabletop centrifuge with a **swing bucket** or **fixed angle rotor** is used, where cell samples are centrifuged in standard (**15 and 50 ml**) sterile plastic tubes. Cells are harvested either as pellets at the **bottom of the tube with a swing bucket rotor** or **at the wall of the fixed angle rotor**.
- Cells are also separated according to size using **density gradient centrifugation** in swing bucket rotor, using **Percoll** or **Ficoll** gradient.

7.1 Rules When Using a Centrifuge

1. Inside the laminar flow hood, the liquid sample is aseptically transferred in sterile **(15 or 50 ml**) plastic centrifuge tubes.
2. If sterile glass tubes are used, they should be protected with a rubber adapter when inside the centrifuge. This will prevent possible breakage.
3. The centrifugation tubes are balanced in a **pan-balance** to make sure that the tubes are of equal weight. In the case of non-balancing, a tube filled with water should be used.
4. The two balancing centrifuge tubes are placed in two opposing slots. If the opposite centrifuge tubes are equal in weight and balanced properly, then only a centrifuge would run smoothly.
5. If the centrifuge is not balanced properly, it will vibrate and can be damaged.
6. Some centrifuges automatically turn off if run in an unbalanced state. Otherwise, centrifuges should be switched off manually as soon as the vibrations occur.
7. The centrifuge is turned on only after closing the safety lid and setting the appropriate speed and time.
8. Once the centrifuge is switched off, the rotor stops after which the safety lid is opened.
9. The cells are pelleted at the bottom of the tube leaving clean supernatant at the top, after centrifugation.
10. Any spills in the centrifuge that might have occurred during centrifugation should be wiped, alongside washing and drying the rotors for every subsequent use.

11. Following centrifugation, the sample cells or tissues can be collected only inside the laminar flow hood to maintain sterility (Shpritzer 2003; Immarino et al. 2007).

8 Sterilization Instruments: Autoclave and Oven

There are two types of sterilization instruments used for mammalian cell culture: autoclave and sterilization oven. A brief discussion of them is as follows:

- Most plastic containers used in cell culture are purchased as pre-sterilized (via UV light treatment) and disposed of, after use.
- All other nonsterile glasswares, glass pipettes, pipette tips, and autoclavable plastic containers used for cell culture must be washed and rinsed with pure water before being sterilized, just before work.
- A small- or medium-sized **oven or autoclave** is sufficient to sterilize the needed material. The oven/autoclave must be operated and maintained following the manufacturer's instructions (Fig. 23).

Fig. 23 External view of a laboratory-scale autoclave

- While the oven uses dry heat to sterilize various materials, the autoclave uses moist heat with high temperature and pressure. So, heat-labile materials such as mammalian cell culture medium cannot be sterilized by both oven and autoclave but by filter sterilization.
- An amicable alternative is the **kitchen pressure cooker**, which could be used for **sterilizing smaller items** (Hugo 1991; Block 2001).

8.1 Autoclave

In 1884, *Charles Chamberland* invented an autoclave. The word autoclave comes from Greek *auto-*, meaning self, and Latin *clavis*, meaning key, collectively inferring a self-locking device. In an autoclave, high pressure and temperature destroy any pathogens, making the cell culture materials germ-free.

8.1.1 Working Principle of Autoclave

- An autoclave is generally used for *moist heating*.
- Contrary to a dry oven that uses high temperature to destroy the pathogens, an autoclave uses moist heat, therefore preventing the degradation of some cell culture molecules that are destroyed by the dry heat of the dry air oven.
- Autoclave works on the basic principle of chemistry, according to which, when the pressure of a gas increases, the temperature proportionally increases. Under these conditions, saturated steam at a *pressure of about 15 psi (temperature around 121 °C/249 °F)*, enters the cells (contaminated microorganisms) and destroys all the macromolecules present inside the cells including proteins. Thereby, all **organisms and their endospores are generally treated** in about 15–20 min.
- At this temperature, all the pathogens including bacteria, viruses, fungi, and particularly the spores of various microorganisms are inactivated.
- Typically, laboratory glassware, heat-resistant plastic containers, other equipment, and surgical instruments are sterilized using an autoclave, regularly.
- As moist heat is used in the autoclave, heat-labile products (such as some plastics) cannot be sterilized to avoid melting and deformation.
- Mammalian cell culture medium and its various ingredients that are sensitive to temperature cannot be sterilized by autoclaving. However, they can be filtered and sterilized.
- For BSL-3 or BSL-4 facilities, pass-through research autoclaves are necessary.
- Sterilization autoclaves are, thus, widely used in research laboratories of microbiology, biotechnology, medicine, veterinary medicine, dentistry, prosthetic fabrication, etc., wherever there is a need for high aseptic-grade materials.
- Medical and biomedical wastes are also routinely destroyed and decontaminated using pass-through autoclaves (Gillespie and Gibbons 1975; Delphine and Barbra 2016).

8.2 Sterilization Oven

- Most laboratories, including mammalian cell culture laboratories, uses hot air oven, the basic principle of which is to use **dry heat sterilization** (170 °C [340 °F] for 60 min, 160 °C [320 °F] for 120 min, and 150 °C [300 °F] for 150 min).
- Generally, glass- and stainless steel-containing materials are the primary choices for sterilization by dry heat.
- The materials that cannot be used in dry heat sterilization are plastics and rubber materials and others that get melted, catch fire, or change their physical states when exposed to high temperatures.
- Surgical dressing cannot be sterilized by these instruments.
- These instruments are not used for wet cell culture or other materials.

8.2.1 Principle of Hot Air Oven

- The dry heat produced by the hot air oven causes sterilization ***via*** the process of conduction. For the conduction of the dry heat in the initial stage, the outer surface of various items absorbs the heat and then transfers the heat to the inner layers (layer-by-layer). Eventually, the temperature reaches all the items and sterilizes them.
- It is recommended that for killing/destroying the difficult-to-kill sporulating microorganisms, an hour of pre-heating is necessary.

NB: The mechanism of dry heat-mediated killing of microorganisms relates to the damage of cells via *oxidizing agents.*

8.3 Types of Hot Air Oven

The static air type of hot air oven
The forced air type of hot air oven

8.3.1 The Static Air Type of Hot Air Oven

In this type of hot air oven, the heating coil is positioned at the bottom. The device works through the rising hot air inside the chamber, via gravity convection. The concerns of this type of sterilization are as follows:

- Slow process and, therefore, requires a long time to reach the sterilization temperature
- Less uniform temperature control throughout the chamber

8.3.2 The Forced Air Type of Hot Air Oven

In this type of hot air oven, the oven works to mediate a forced-air or mechanical convection sterilization. It is equipped with a motor-driven blower that circulates hot air throughout the chamber at a high velocity. Thus, this kind of instrument allows a rapid transfer of energy from the air to the periphery.

8.4 The Advantages of Dry Heat Sterilization

- This type of machine is very easy to install.
- There is almost no operating cost.
- Dry heat easily penetrates various items.
- No toxicity is generated by operating this type of instrument and, therefore, there is minimal environmental risk.
- It is noncorrosive for metal and sharp instruments.

8.5 The Disadvantages of Dry Heat Sterilization

- Time-consuming method because of slow heat penetration inside the microbial body.
- Most of the mammalian cell culture materials including cell culture medium get destroyed by this technique since they contain multiple temperature-sensitive constituents such as amino acids, growth factors, hormones, etc.

9 Temperature-Controlled Water Bath

- A *water bath* is a laboratory equipment made from a container filled with heated *water* (Fig. 24).
- It is used to incubate samples in *water* at a constant temperature over a long period. All *water baths* have a digital or an analog interface to allow users in setting the desired temperature.
- All mammalian cells are incubated in a CO_2 incubator at 37 °C.
- Therefore, for thawing of frozen cells, warming of cold buffers and medium (cell culture medium is stored at 4 °C until the date of expiry, and complete medium

Fig. 24 Snapshots of (**a**) a circular and (**b**) broad interface (for sample keeping) water baths

with all the additives is kept in 4 °C refrigerator up to 1 week) is conducted on a water bath at 37 °C.
- In this way, the cold shocking of cells can be avoided.
- Special care should be taken so that the screw cap of the reagents and medium containers does not come in contact with the water bath. Bottles and containers after warming up in a water bath are **wiped carefully with 70% alcohol** before being transferred to the laminar flow hood.

*NB: The microorganisms, particularly bacteria and fungus, and other contaminants can grow in warm water inside the water bath. So, the water bath needs to be periodically cleaned and the used water needs to be replaced with fresh distilled water (if possible sterile). Fungicides (*e.g.*, amphotericin B) can also be added to the water bath to prevent any fungal growth.*

10 Water Purification System

- Water is used in many steps of the tissue or cell culture processes.
- Ultrapure or deionized water is the main component of buffers and cell culture medium; it is used for the dissolution of additives and drugs and the water container in a CO_2 incubator. Single distilled water can be used for rinsing bioreactors, plasticware, and glassware.
- Many other instruments such as autoclaves, incubators, etc. also use water.

Some compounds naturally present in water that can affect cell culture are as follows.

10.1 Bacteria and Inorganic Ions Present in the Water

- The nonpathogenic or even pathogenic bacteria may be present in the water, causing a change in the pH of the water and releasing endotoxins such as lipopolysaccharide (**LPS**).
- Changes in the medium pH, as well as endotoxins, affect the cell growth and function, cloning efficiency, and production of recombinant proteins.
- Several heavy metals may be present in the water such as Pb, Hg, Cr, Zn, Cd, and Ni. Experimentally, the heavy metals show toxic effects on the cultured mammalian cells including glial and neural cells.
- A high concentration of magnesium affects Taq DNA polymerase in PCR reaction. Additionally, magnesium inhibits glucose-6-phosphate dehydrogenase and DNA nuclease II.
- Manganese causes DNA polymerase to slot in ribose instead of deoxyribose inside the nascent DNA chains.

Fig. 25 Laboratory-scale water purification system for deionized water supply

10.2 Organic Compounds Present in the Water

- The small organic molecules that may be present in the water are pesticides, tannins, humic acids, etc.
- These organic substances are known to affect the mammalian cell culture.

Thus, because of all the abovementioned impurities, water quality may play an important role in cell culture experimental outcomes (Fig. 25).

11 Refrigerators, Freezers, and Deep Freezers

All the laboratories use several nonvolatile chemicals, biomaterials, etc. that are temperature sensitive. These kinds of materials need to be stored at various temperatures. For example, cell culture medium needs to be stored in refrigerators, restriction enzymes, DNA, antibodies, etc. in freezers, and RNA needs to be stored in a deep freezer. Thus, these instruments are essential for all mammalian cell culture laboratories.

The temperature range of refrigerators, freezers, and deep freezers is as follows:

Refrigerators: ranges from +2 °C to +15 °C
Freezers: ranges from −10 °C to −24 °C
Deep freezers: ranges from −80 °C to −150 °C

These instruments are necessary for storing various materials and reagents needed for the cell culture.

For example, while both liquid and lyophilized dry cell culture medium are stored inside 4 °C refrigerators, antibiotics and serum are generally stored at −20 °C (Fig. 26).

Similarly, cells should be frozen (temporarily) between −80 °C and −150 °C only.

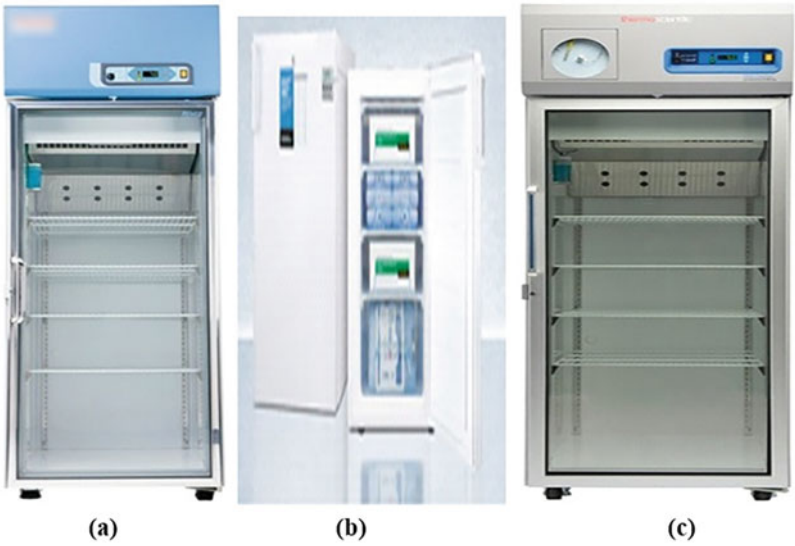


Fig. 26 Laboratory-scale refrigerators workable at (**a**) 4 °C, (**b**) −2 °C, and (**c**) −80 °C

Constant temperature refrigerators are developed to meet the need for 2–8 °C storage of vaccines, insulin, and other small-volume temperature-sensitive materials. They can be mapped with temperature sensors throughout and often meet the USP validation requirements (with ambient room temperatures of 60–80 °F).

The chromatography refrigerator generally maintains 5.5 °C and is used for protein purification.

12 Liquid Nitrogen Facility

Liquid nitrogen containers are necessary for step-by-step cryopreservation of mammalian cells. The stored cells bear a complete chance of recovery at any time (even years) as long as they are stored properly with an uninterrupted liquid nitrogen supply.

12.1 Liquid Nitrogen Containers and Cryopreservation of Cells

- Liquid nitrogen containers are special containers for keeping liquid nitrogen. Each container contains several vessels/racks (Fig. 27).
- In each rack, pre-marked special boxes are kept in which vials of frozen cells are preserved at ultra-low temperature (**−196 °C**).
- This grid-like position of the frozen cell vials in the box indicates a racking inventory system designed to organize the contents for ease of location and rapid cell vial retrieval from liquid nitrogen containers.

Fig. 27 Laboratory-scale liquid nitrogen cooling facility

- This includes proper documentation of all cell vials through accurate record-keeping and inventory control.

During maintenance of the frozen cell repository in liquid nitrogen, the following directions are followed:

- Each vial containing mammalian cells should be individually labeled with the name of the cell line, lot number, and date of freezing.
- The location of each cell vial should not only be recorded, ideally as a soft copy in a spreadsheet but also as a hard copy like conventional paper inventory.
- There should be a well-built inventory system to make sure that no cell vials can be deposited or taken out without updating the records.
- It is imperative that staff using the liquid nitrogen facility should be well trained and follows necessary safety precautions about liquid nitrogen exposure and skin burning during regular filling of containers.
- Protective garments like special thermally insulated gloves and goggles should be worn at all times while handling nitrogen.

- Each laboratory personnel must wear a laboratory coat or preferably a splash-proof plastic apron, to have protection against accidental spillage of nitrogen.
- There is a potential hazard of asphyxiation (in the staffs) owing to the vaporization of escaped liquid nitrogen that displaces atmospheric oxygen.

NB: The cryopreservation procedure is described in chapter ▶ "Mammalian Cell Culture Types and Guidelines of Their Maintenance."

13 Biohazard Waste Containers and Sharp Containers

- When working in the mammalian cell culture, laboratory biohazard wastes are generated. The biohazard waste would be placed in different kinds of biohazard waste containers before being disposed of safely.
- It is recommended that for the handling of biohazard materials, government and institutional regulations must be followed.
- To prevent the environmental spread of potentially hazardous materials, complying with the rules is very important (Fig. 28).
- **Liquid waste** produced by the cell culture medium can be aspirated directly to the disinfectant inside the vacuum trap container and kept at least 20 min before being disposed of.
- The cardboard biohazard pouches or plastic biohazard containers are used to keep **pipette tips, disposable glass pipettes,** and any other sharp materials. They should be autoclaved before disposal.
- The cylinders filled with disinfectant are used to keep **glass serological pipettes**. Wash, sterilize, and reuse these materials.
- Boxes or cylinders can be **used to temporarily store disposable serological pipettes** and are sterilized before disposal.

Fig. 28 Biohazard discharge provision in a laboratory setup [figure is a snapshot of our laboratory provision]

- **Glass and surgical blades, needles, etc.** are disposed of after disinfecting the special glass biohazard containers. Cotton with blood samples or cells or medium, etc. should be disposed of separately after being sterilized.

14 Conclusions

A mammalian cell culture laboratory is equipped with various instruments that are required for several direct and indirect activities related to the culture of mammalian cells and their maintenance. **Broadly, mammalian cell culture facility is divided into three parts: main cell culture laboratory, anti-room, and outer laboratory**. The main cell culture laboratory is the most important part of mammalian cell culture. The instruments present in the main cell culture laboratory are utilized not only for culture but also for the maintenance and propagation of mammalian cells. Since microbial contamination is the major problem of mammalian cell culture, efforts are taken in designing the mammalian cell culture laboratory to keep it as germ-free as possible. So, an anti-room is set up just outside the main cell culture facility. The anti-room is a buffering zone just before entering the main cell culture facility to minimize the passage or transfer of dust particles/microorganisms carried by the laboratory personnel. This anti-room is utilized for keeping shoe racks and storage for disposable, protective dresses. The laboratory personnel should wear laboratory coats and personal protective equipment (PPE) before entering the main cell culture laboratory. A HEPA filter room and a shower room are located adjacent to the anti-room. At the periphery of the anti-room, the outer laboratory is located which is occupied with various accessory instruments necessary for mammalian cell culture. The main available instruments in the outer laboratory are equipment for sterilization and washing (autoclave/oven, washing sink, etc.), facilities for medium preparation (chemical storage self, balance, water purification system, water bath, refrigerators, etc.), maintenance of cultured cells(−20 °C and −80 °C deep freezers, liquid nitrogen containers, etc.), and storage of cell culture grade, disposable sterile containers (culture plates, flasks, tubes, pipettes, etc.). Common safety instruments (e.g., eyewash station) and biohazard disposal containers are also kept in the outer area. *The chapter describes all these instruments and their usefulness in mammalian cell culture.*

15 Cross-References

► Establishment of a Cell Culture Laboratory
► Mammalian Cell Culture Types and Guidelines of Their Maintenance

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Common Reagents and Medium for Mammalian Cell Culture

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_4-2

Abstract

This chapter describes all common materials and reagents necessary for mammalian cell culture. Mammalian cells are normally present in tissues and organs. These cells attach to the extracellular matrix (**ECM**). So, **cell-dissociating agents** are highly essential to detach cells from the tissues/organs. Most mammalian cells are adherent in nature, except for some blood cells (e.g., B and T lymphocytes). The next important material needed for mammalian cell culture is **cell adhesive**

agents which are extremely essential for the attachment of mammalian cells with culture vessels (Petri plate/flasks). The most important material necessary for mammalian cell culture is the **medium** and its various necessary constituents. A complete cell culture medium contains all the necessary materials needed for the proliferation and growth of mammalian cells such as carbohydrates (e.g., glucose), protein/peptides (e.g., amino acids), lipids (fatty acids and glycerol), minerals, vitamins, hormones, growth factors, cytokines, and other materials. One of the most important components of the cell culture medium is a **fetal bovine serum** (**FBS**) or **fetal calf serum** (**FCS**) which contains many of the constituents of the mammalian cell culture medium. However, low serum or serum-free media are also used in particularly large-scale cell cultures for industrial purposes. The need for the above-mentioned materials and their relative quantity may vary from one type of cell to another, and therefore, **various types of cell culture** media have **been developed** with varying compositions. Finally, **various buffers** necessary for isolation, culture, and maintenance of mammalian cells are also mentioned.

Keywords

Cell dissociating agents · Cell adhesive agents · Buffers for cell culture · Cell culture medium · Fetal bovine serum (FBS) · Fetal calf serum (FCS) · Serum free medium · Antibiotics · Antimycotics

1 Introduction

Several studies including that of Carrel explained the requirements of various materials for in vitro culture of tissues (Carrel 1912). Several materials are needed for mammalian cells isolation from various tissues and organs and subsequent culturing. In a mammalian tissue or organ, various cells are joined together by extracellular matrix (ECM) proteins. Of the various proteins present in the ECM, structural protein collagen (triple helix protein) and adhesive proteins, fibronectin and laminin are highly important. To separate cells from tissues or organs, it is highly essential to dissociate or break collagen, fibronectin, and laminin that help in ECM-cell surface joining. Based on the type of tissues or organs, either mechanical disaggregation or enzymatic dissociation of cells is necessary. Cell-dissociating agents such as various proteolytic enzymes (e.g., collagenase, trypsin) and their usefulness are discussed in this chapter.

Once the cells are isolated from tissues and organs, the next important materials needed for cell culture are containers (vessels) of cell culture and cell adhesive agents. Special types of polystyrene-made sterile cell culture containers and vessels, plasticware, as well as different heat-resistant autoclavable glassware, transfer aides, and membrane filters, are extensively used in mammalian cell culture.

Except for some blood cells (e.g., leukocytes), all other cells in the mammalian body are adhesive. Since blood cells are suspended in the plasma, it is easier to

isolate them by a range of procedures including differential centrifugation. Adhesive cells grow in attached mode with the cell culture containers for their survival and growth. Various cell adhesive agents like gelatin, laminin, etc. are especially needed for the culture of all adhesive cells. For harvesting and subculturing adhesive cells from the cultured containers, trypsin-EDTA is used, which would remove the cell from the culture containers. However, for trypsin-sensitive cells, a cell scraper can be used. Nonadhesive cells such as B or T lymphocytes grow in suspension and therefore do not require any adhesive agents for their culture medium (Fig. 1).

Besides cell-dissociating and adhesive agents, another important material discussed in this chapter is the cell culture medium. Medium is the artificial environment in which mammalian cells survive and grow. A cell culture medium comprises all the nutrients including glucose, amino acids, hormones, growth factors, vitamins, inorganic salts, etc. needed for mammalian cell survival and growth. (5–10)% fetal bovine serum (**FBS**) or fetal calf serum (**FCS**) is also generally supplemented to the cell culture medium for growth and proliferation. However, serum may inhibit the proliferation of some cells. Also, the serum is one of the most expensive components of the cell culture medium. Therefore, the serum-free medium is generally utilized for some mammalian cell culture as well as industrial-scale cell culture volume, where the cost of the cell culture products is highly important. Additionally, the need for nutrition varies from one to another cell type, including some cells that require special nutrition for their growth. For example, endothelial cells grow well in the presence of vascular endothelial growth factors (**VEGFs**). Medium not only supplies nutrition but also maintains pH, osmolarity, temperature, and moisture alongside the O_2/CO_2 tension of the cells.

Finally, several buffers are utilized for various purposes in mammalian cell culture. Some **common buffers** such as phosphate buffer saline (**PBS**), Hanks Balanced Salt Solution (**HBSS**), etc. are widely used for washing mammalian cells and cell culture containers. This chapter discusses all the above-mentioned subjects/materials necessary for mammalian cell isolation and culture.

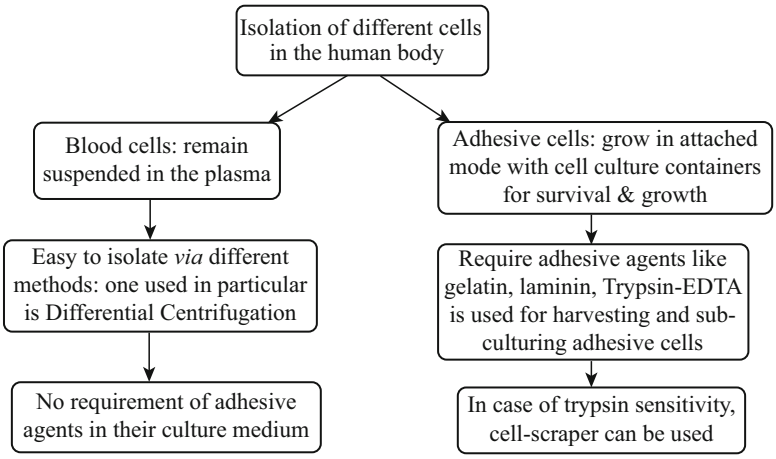

Fig. 1 Isolation distinctions of adhesive and suspended cells for their subculturing

2 Physiological Parameters Necessary for the Culture of Mammalian Cells

Besides an adequate supply of common materials for the isolation and culture of mammalian cells, ideally, some physiological parameters are also needed to be strictly maintained for mammalian cell culture.

2.1 Culture Vessels with Surface Property

Proper cell culture plates or flasks made of **polypropylene/polystyrene** are used for the growth of adherent mammalian cells. Of note, the mammalian cell membrane possesses a net negative change in its surface. Nonadherent cells such as leukocytes (e.g., B/T cells) grow in suspension.

2.2 Composition of Cell Culture Medium

Medium with essential nutrients, minerals, vitamins, amino acids, growth factors, hormones, etc. are appropriate for culturing particular mammalian cells.

2.3 pH of the Cell Culture Medium

In general, the cell culture medium should have a physiological pH between 7.2 and 7.5 (average 7.4 which is the pH of the human blood). This pH is maintained either via HEPES buffer and/or sodium bicarbonate addition. Cells should be kept in an incubator in which the gas phase of 5% CO_2 tension and bicarbonate in the medium are kept in equilibrium (Howorth 1975).

NB: Human blood pH is 7.4 and human cell cytoplasm pH is 7.2. Human cell cytoplasm maintains a reducing environment.

2.4 Temperature and Light

All mammalian cells in the culture medium are maintained at 37 °C in a CO_2 incubator usually in a dark environment (**no light or illumination is necessary**).

2.5 Humidity of the CO_2 Incubator

The CO_2 incubator maintains nearly 95% humidity, which is needed for mammalian cell culture. Humidity is maintained at a residual **95% of sterile air** either using sterile water or via *a* water-jacketed CO_2 incubator.

2.6 Subculture and Feeding with Culture Medium

Depending on the cell type, when cells are divided to optima and culture medium is spent, cells are again harvested, spent medium is discarded, and fresh medium is added which is called the **subculture** of cells. If the culture vessels are full, the cells will be split into more culture vessels. For example, cells harvested from one 75 mm flask may be divided into two to three 75 mm flasks. Sometimes, subcultures are done either once or twice per week. Many times, spent medium is discarded in between and fresh medium is added gently without disturbing the cells. This procedure is also called as **feeding** of cells (Masters and Stacey 2007).

3 Cell Dissociation Agents

- Mammalian cells are isolated from their tissues and organs. Smaller tissues or organs could be cultured directly, even as **explant culture**.

NB: Explant culture is not popular and requires lots of standardization besides varying from culture to culture.

- Depending on the cell types and nature of experiments, different cell-dissociating reagents are used for both isolation of cells from tissues and organs in primary culture as well as detachment of adherent cells from the surface of culture plates or flasks (during subculture). Generally, Trypsin-EDTA is used for the detachment of cells from the cell culture containers.
- The molecules or agents that dissociate cells from the tissues or organs are called **cell-dissociating agents**. These agents are used for aseptically dissociating cells from complex mammalian tissues and organs, after dissection. Once dissociated, these cells remain suspended in a culture medium and are called **single-cell suspension**. These single-cell suspensions are allowed to grow in vitro within a CO_2 incubator.
- Either mechanical disaggregation or different types of cell-dissociating agents are used depending upon the used cells, tissues, and organs. Some dissociating agents are **proteolytic (enzymes that digest the proteins, e.g., trypsin)** and **collagenolytic (enzymes that digest the matrix triple helix protein, e.g., collagenase type 1 from *Clostridium histolyticum*)** enzymes while others are active in a nonenzymatic salt solution. The isolation of various cells from tissues and organs and the primary cultures, such as endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells is discussed in chapter 08.
- Also since most mammalian cells are adherent in nature, they will attach to the polystyrene-coated cell culture containers before their growth. For harvesting or subculturing, the cells must be detached. When cells adhere to the surface of the culture flask or plates, the dissociating agents are again used to detach these cells from the surface before being pooled in suspension. This technique is called as **harvesting of cells**. The most abundantly used proteolytic dissociating agents are

0.25% or **0.01% trypsin**. EDTA solution is used as a nonenzymatic cellular dissociation agent. **EDTA is used with or without trypsin to prevent metallic toxicity to the cells**. The source(s) of the metal constituents may be medium constituents or culture vessels (Allen and Snow 1970; Dalen and Todd 1971; Heng et al. 2009).

4 Cell Adhesive Agents

- Most mammalian cells are adhesive, so attachment of these cells to the cell culture containers is a prerequisite for the ideal growth.
- Thus, for culturing adherent cells, culture containers must be pre-coated with various adhesive agents or matrix adhesion proteins.
- Most commonly used adhesive agents are ***gelatin, collagen, fibronectin, laminin, poly-L-Lysine,*** etc.
- Gelatin is the **most common** and cheapest adhesive used in endothelial and other adhesive cell cultures.
- Matrix adhesion proteins are responsible for linking the matrix components to one another and the cell surfaces. They interact with collagen and proteoglycans to specify matrix organization and are the major binding sites for **integrins** (Curtis 1962; Curtis et al. 1983; Ramsey et al. 1984).

Here is a brief discussion of the two most important matrix adhesion proteins, that is, fibronectin and laminin.

4.1 Fibronectin

- Fibronectin is an adhesive glycoprotein that prevails present in the ECM. This protein exists in both soluble secretary form (liver) in the plasma as well as insoluble principle adhesive texture of connective tissues. It is also expressed as a cell surface protein. Initially, it is produced as a preprotein before being finally processed to form a mature protein.

Here are the brief structural and functional details of fibronectin.

4.1.1 Structure of Fibronectin Protein

- While fibronectin is produced by a single gene, several alternative splice variants of this gene are detected.
- Fibronectin is recognized **as a** high molecular weight (MW) glycoprotein, with the MW ranging from ~ (440–530) kDa.
- This protein exists as a **dimer of two nearly identical polypeptide chains linked by a pair of C-terminal disulfide bonds**.
- Each polypeptide chain is made up of nearly 2500 amino acids, having the MW within ~ (230–275) kDa.

- The polypeptide functional fibronectin domains could be of a structurally distinct type, namely, FN1 (I), FNII (II), and FNIII (III) repeating subunits (Fig. 2).
- All three fibronectin domains consist of antiparallel β strands with conserved residues in their hydrophobic cores.
- The binding of fibronectin to integrins (transmembrane adhesive protein with bidirectional signaling, inside out and outside in) unfolds fibronectin molecules, forming the dimers, which can function properly.

4.1.2 Functions of Fibronectin

- This protein is implicated in several functions, including the most prominent of which is **adhesion**. At present, fibronectin is recognized as a **classical cell adhesion molecule and is regarded as a master organizer of ECM**. It interacts or cross-talks with **collagen**, **fibrils**, **and GAGs**.
- **Reorganization of the cytoskeleton** is another important function of fibronectin. The binding of fibronectin with collagen and cell surface integrins reorganizes the cytoskeleton of the cells besides facilitating cell movement.
- The other well-recognized functions of fibronectin are oncogenic transformation, cell migration, phagocytosis, hemostasis, and embryonic differentiation.
- It is now well accepted that fibronectin has a role in wound healing via binding to platelets at the site of tissue injury apart from the cellular movements during wound healing.

4.2 Laminin

Laminin is a glycoprotein of ECM that interacts with other plasma membrane proteins/receptors located at the basal lamina. The major function of this protein is adhesion.

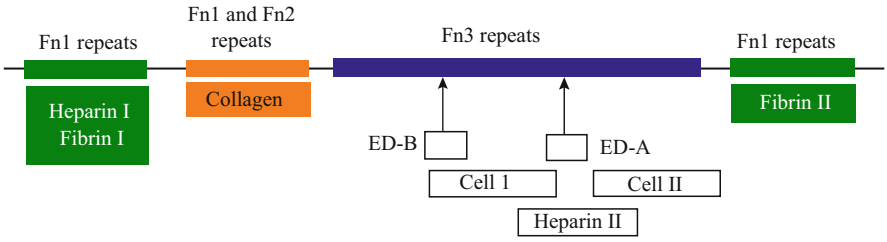

Fig. 2 Typical representation of a fibronectin polypeptide chain, comprising of multiple repeat elements (Fn1, Fn2, and Fn3) and binding sites for multiple matrix proteins and cells. Two regions can bind heparin and fibrin while the other two are involved in cell binding. ED-A and ED-B are the isoforms generated by alternative splicing which may or may not contain certain Fn3 domains. Other splice variations in the second cell-binding domain generate distinct isoforms

Here is a very brief discussion of the structure and functions of laminin.

4.2.1 Structure of Laminin

- Laminin is a very high MW (~900 kDa) glycoprotein. This complex protein is a heterotrimer of α, β, and γ subunits.
- Further, these subunits are the products of five α, four β, and three γ genes.
- The α, β, and γ chains are linked together in a cruciform structure (with globular and rod-like domains) stabilized via disulfide linkages (Fig. 3).
- So far, 15 laminin isoforms have been identified.
- Like other matrix proteins such as type IV collagen, laminins can self-assemble into mesh-like polymers.

4.2.2 Functions of Laminins

- The major functions of laminins are cell adhesion and cell-to-cell interaction.
- To fulfill these functions, laminin interacts with various proteins such as cell surface receptors integrins, type IV collagen, heparin sulfate proteoglycan (a perlecan), entactin, and other molecules, forming a cross-linked network in the basal lamina.
- Laminin may have a role in neural development and peripheral nerve repair.

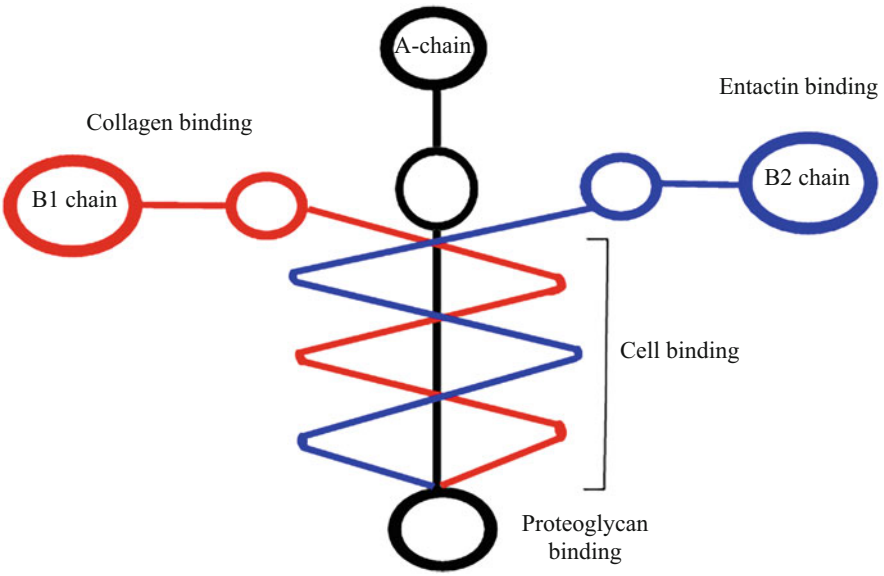

Fig. 3 Multimeric binding domains of laminins, characterized by A, B1, and B2 polypeptide chains (nowadays referred to as α, β, and γ chains), which are held together in a cruciform structure stabilized by disulfide linkages

4.3 Cell Adhesion to the Extracellular Matrix

- Many cells (plasma membrane) bind to the ECM components.

Cell adhesion can occur in the following two ways:

4.3.1 Focal Adhesions

It is a process of connecting the ECM to actin filaments of the cell.

4.3.2 Hemi-Desmosomes

These molecular structures are involved in connecting the ECM to intermediate filaments such as keratin.

- **Integrins**, a family of transmembrane proteins perform highly important roles in cell-ECM cross-talk-dependent adhesion. This specialized protein sends signals bidirectionally, from outside the cell to inside and from inside the cell to outside (commonly referred to as inside out and outside in).
- This protein consists of α and β subunits. Various types of α and β subunits joined together to form at least 24 different integrins.
- **Fibronectins and laminins** bind to ECM macromolecules and facilitate their binding to transmembrane integrins.
- ***Integrins facilitate binding with several cytoskeleton proteins such as vinculin, paxillin, talin, and focal adhesion kinase (FAK), maintaining the structural integrity of the cells*** (Fig. 4).

NB: Collagen, fibronectins, and laminins are natural proteins comprising the mammalian ECM.

- **Gelatin** is a derivative of mammalian collagen.
- **Poly-L-Lysine** is a positively charged synthetic protein that was originally produced from bacteria.

Gelatin and Poly-L-Lysine are also used as cell adhesive agents. They are used for coating the cell culture containers.

5 Cell Culture Medium and its Constituents

- **Cell Culture Medium** is an artificial environment that comprises essential nutrients, amino acids, vitamins, minerals, growth factors, etc. for supporting cell growth and proliferation by providing optimum nutrition.
- The cell culture medium is the artificial environment that not only supplies all the nutrients necessary for the culture and growth of mammalian cells but also maintains the pH, temperature, moisture, and O_2/CO_2 tension of the culture environment.

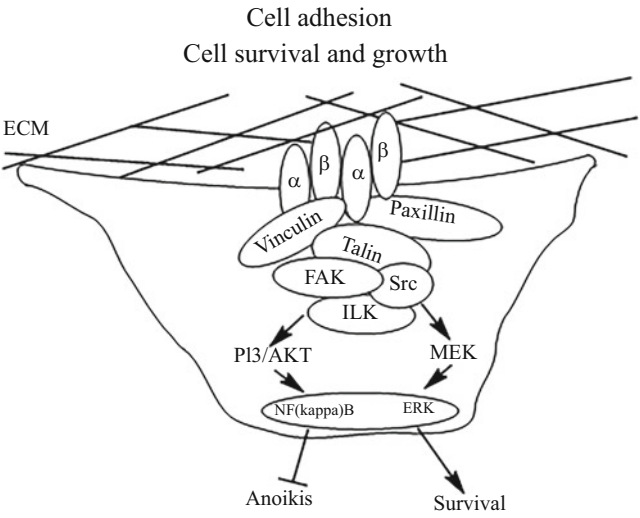

Fig. 4 Representative scenario of ECM-bound cell growth, where α and β subunits of integrins play key roles in keeping the cells glued to matrix proteins and gap junctions. The sole mandate of such a growth pattern pertains to regular nutrient intake from the matrix alongside regular communication with surrounding cells. Cells devoid of basal membrane growth support are designated as following **Anoikis** (characteristic of tumor cells)

- Every cell, including those of immortal tumor or cancer cells, needs the culture medium for its proper growth and development. However, the need for the various cell culture medium nutrients may vary from one to another cell type. Thus, cell culture medium composition varies for different cells.
- The choice of a specific medium and its supplements depends upon the grown cell type and its specific origin. For cells from special tissues needs, more complex culture medium is needed with special supplements, such as **VEGFs** are needed for endothelial cell culture.
- When ingredients of culture medium for a specific cell type are not known, different culture media with growth factors and serum are tested for optimum results by monitoring their growth microscopically.
- Cell culture medium helps to maintain the pH (7.4–7.5), temperature (37 °C), and moisture and humidity (95%) for the best growth of cultured cells (White 1946; Fischer et al. 1948; Morgan et al. 1950; Parker 1961).

5.1 Composition of Cell Culture Medium

As mentioned above, the cell culture medium maintains every factor responsible for the proper growth and maintenance of mammalian cells, including nutrition, pH, and osmolarity. Many of the contributions to designing the nutritional composition of the mammalian cell culture medium were initially established separately by *Carrel and Eagle* (Carrel and Baker 1926; Eagle 1955).

The specific composition of a cell culture medium is based on the following four important functions:

- To supply necessary nutrition to the cultured cells
- To maintain pH of medium/cultured cells
- To maintain osmolarity of the medium/cultured cells
- To provide and maintain proper temperature, moisture, and O_2/CO_2
- Tension of the mammalian cells using a CO_2 incubator

The following paragraphs provide a brief discussion on the nutritional composition, pH, and osmolarity maintenance of the cell culture media.

5.2 Nutritional Composition of the Cell Culture Medium

Cell culture medium contains the following materials.

5.2.1 Carbohydrates

- Biochemically, carbohydrates are called **poly-hydroxy-aldehydes** and **ketones.** Carbohydrates can be divided into **monosaccharides, disaccharides** (two molecules of monosaccharides joined via glycosidic linkage), and **polysaccharides** (more than two molecules of monosaccharides joined together via glycosidic linkage).
- Carbohydrates are one of the most important energy sources. Disaccharides such as fructose and polysaccharide such as glycogen cannot be metabolized by mammalian cells. Therefore, they must be broken into monosaccharides before their metabolism inside the cells.
- Intestinal cell absorptive capacity varies among different monosaccharides with the highest for glucose and the least for galactose. **Glucose** is the most abundant monosaccharide in the mammalian blood, which is transported to the cells and subsequently metabolized to produce energy (ATP), and therefore is evidently the most **common sugar (used as a carbon source)** in the medium.
- Depending upon the cell type, the culture medium may have distinctive glucose contents.
- Some media may contain **dextrose** (a monosaccharide), **galactose** (a monosaccharide), **fructose** (a monosaccharide), and **maltose** (a disaccharide, consisting of two glucose molecules).
- **These monosaccharides are isomers of glucose and convert to glucose before metabolism and releasing energy (ATP/GTP)** (Lewis 1922).
- Sometimes, in addition to glucose, sodium pyruvate, a metabolic product of glycolysis (in the mammalian cell cytoplasm one glucose molecule is converted to two pyruvic acid molecules), oxaloacetate, alpha-ketoglutarate, etc. (Neuman and McCoy 1958) is freshly added to the medium to ensure an easier energy supply or even cyclic GMP (cGMP) may also be added (Seifert and Rudland 1974).

5.2.2 Amino Acids

- A protein is a polymer of amino acids joined together by a peptide bond (a type of covalent bond). In other words, amino acids are the smallest constitutional units of a protein.
- Structurally, an amino acid consists of an amino group (-NH_2), a carboxylic group (-COOH), and a side chain (R-CH).
- So, the basic formula of an amino acid is NH_2-RCH-COOH.
- The smallest and simplest amino acid is glycine with a formula of NH_2-CH_2-COOH, where side chain R is replaced by hydrogen.
- During peptide formation, the amino group of an amino acid joins with the carboxylic group of another amino acid to form a peptide bond (CONH), releasing one molecule of water (H_2O).
- In the cellular system, nitrogen released by an amino acid/protein is used to form nitrogenous bases such as purines and pyrimidines.
- Without purine and pyrimidine bases, DNA and RNA cannot be synthesized (replicated and transcribed, respectively). Additionally, without replication, transcription cell division or proliferation is not possible, amino acids are added as obligatory ingredients of all known cell culture media.
- Although in nature *around **300 amino acids** have been discovered so far, only **20 amino acids are involved in peptide bond formation and thus protein synthesis***.
- **Glycine** is the simplest amino acid in the human body.
- While some amino acids are synthesized by the mammalian cells, others cannot be and therefore must be supplied through food, called **essential amino acids**.
- The **twelve essential amino acids** that must be supplied through foods are arginine, cysteine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, histidine, tyrosine, and valine. All these amino acids are **L-amino acids**.
- During mammalian cell culture medium preparation, special attention should be given to amino acid **glutamine**, since it is necessarily needed to synthesize nitrogenous bases for DNA and RNA.
- **The L-glutamine** concentration varies from one another culture media. For instance, in M-199 media, it is **0.68 mM** and for Dulbecco's Modified Eagle's Medium (DMEM) it is **4 mM**.
- **NB:** A proper concentration of amino acids in the mammalian cell culture medium must be maintained, failing which proper cell growth would not be possible. On the other hand, concentration higher than the optimum concentration may produce a large quantity of ammonia which is toxic for mammalian cells.
- Besides essential amino acids, several nonessential amino acids are added to the mammalian cell culture medium, since these may be depleted during cell culture.
- Supplementation of a mammalian cell culture medium with nonessential amino acids stimulates growth and prolongs cell viability (Hosios et al. 2016).

NB: The amino acid L-glutamine is easily degraded and is, therefore, generally added freshly in the culture medium.

5.2.3 Peptides and Proteins

- Besides amino acids, proteins and peptides can be directly added to the cell culture medium.
- The most commonly used proteins and peptides are **albumins, transferrin, fibronectin, aprotinin,** and **feutin** (Fig. 5).

Human Serum Albumin

Human serum albumin (**HSA**) is one of the most important serum proteins that not only maintains blood **osmolality** but also binds with various molecules such as steroid hormones and acts as their carrier in the mammalian body. HSA is used to eliminate the toxic substances in the cell culture medium by binding to them. It also stabilizes various small molecules (including administered drugs) by binding to them and safely delivers them to the cells.

NB: Since HSA shows antioxidant activities, it cannot be used in the mammalian cell culture medium as and when the potential anti-oxidant activities of an unknown or newly developed compound are the experimental purpose.

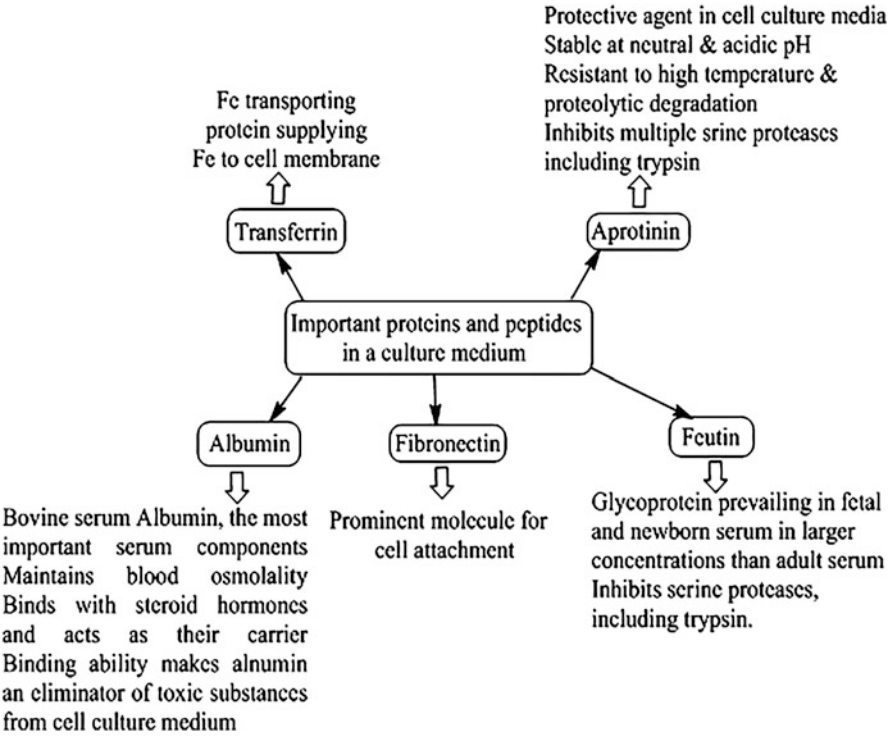

Fig. 5 Important functions of some proteins and peptides included in different culture media

Aprotinin

- It is the protein used in mammalian cell culture media to destroy the effect of various proteases that may be released by the injured or apoptotic cells.

Fetuin

- The name fetuin designates a protein released in the circulation because this glycoprotein is produced by the fetus and newborn baby. However, a detectable but very low concentration of fetuin is also released in adult human beings. Just like aprotinin, fetuin also destroys proteases, particularly serine proteases released by the injured or apoptotic cells.

Fibronectin

- As discussed in the previous sections, fibronectins are added to the mammalian cell culture medium to facilitate the attachment of adherent cells to the culture containers.

Transferrin

- Transferrin is a plasma glycoprotein that is utilized to transport iron (two molecules of ferric iron per transferrin) in the cells. This protein is almost exclusively produced by the liver.

NB: These proteins may be added to the serum-free medium. However, in the serum-containing cell culture medium, there is no need to add these proteins.

5.2.4 Fatty Acids and Lipids

- Generally, a serum-containing medium does not require fatty acids and lipids, as they are generally present in serum.
- They are particularly important in serum-free medium.
- **Linoleic, oleic, and arachidonic acids** are important fatty acids associated with albumins in nature.
- **Albumin** helps in improving the solubility of linoleic, oleic, and arachidonic acids as well as their oxidative protection.

5.2.5 Minerals and Trace Elements

- Various minerals and trace elements (micronutrients) are present in human circulation, which are necessary for various biological functions including the functioning as a cofactor for various enzymes or proteins and vitamins, pattern of glycosylation (a type of posttranslational modification which enzymatically adds carbohydrates to protein), protein folding and unfolding, and finally, growth and proliferation of mammalian cells.
- It is discovered that around **21 minerals** that are needed for various biological functions are not produced by the human body and therefore are called **essential minerals**. Therefore, deficiency of one or more of these elements leads to pathophysiological conditions.

- Out of these 21 minerals, the most important trace elements that affect the culture, growth, and bioprocessing process are **zinc, aluminum, manganese, molybdenum,** and **iron**. To some extent, a second category includes **copper** and **nickel**. It is experimentally observed that all these elements are necessary for the glycosylation of protein.
- Other minerals that are not only necessary for maintaining the osmolarity of cells/plasma, but also for various biological functions are **sodium, potassium, calcium, chlorine, phosphorus, bicarbonate, zinc, selenium, and copper**.
- The most common minerals that exhibit deficiency in humans are **calcium, iron, and iodine**.

NB: Since serum contains all the mineral and trace elements, there is no need for the addition of these materials in the mammalian cell culture medium, except in a serum-free medium. Various companies use the serum-free medium in their mammalian cell culture-based product development since the serum is a cost additive for cell culture medium (Eagle 1956; Cinatl 1969).

5.2.6 Vitamins

- Vitamins are a type of micronutrient that is not produced by the human body and are therefore called essential micronutrients. There are **13 essential vitamins**.
- While some vitamins are water-soluble (e.g., vitamin B complex and vitamin C), others are fat-soluble (e.g., vitamins A, D, E, and K).

  Vitamins are supplemented through various foods.
- The common vitamin components of any culture medium are **biotin, folate, nicotinamide, pantothenic acid, pyridoxine, riboflavin, thiamine, and vitamin B12**.
- Vitamins mainly act as **coenzymes or prosthetic groups** in cell metabolism and therefore are necessary for mammalian cell growth and proliferation.

NB: In general, since mammalian serum contains all vitamins, there is no need to separately supply vitamins to the cell culture medium unless special attention is needed because of an enhanced need for certain vitamins.

5.2.7 Hormones

- Hormones are the biochemical messengers produced by the ductless **endocrine glands** of a mammalian body. Being secreted by an endocrine gland, hormones diffuse into the blood and reach various target sites through the blood.
- The major biochemical nature of the hormones are **peptides** (e.g., oxytocin/vasopressin), **proteins** (insulin/glucagon), biological **amines** (e.g., thyroxine), and **steroids** (estrogens/testosterone).
- The hormones are involved in growth, metabolism, sexual characteristics, and other functions.
- Of note, various hormones have distinct biological functions.
- Some cell culture media may require hormone addition from outside.

- Hormones like **insulin, cortisol, estrogen**, etc. may be added in different cell culture media. For example, the culture of MCF-7 breast cancer cells requires estrogen for their proliferation.

5.2.8 Growth Factors

- Growth factors are small proteins or peptide molecules that induce cell division or proliferation and are, therefore, **mitogenic**.
- Various mammalian tissues produce growth factors, many of which are secreted in the blood.
- Some important growth factors discovered to date are **vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), neuronal growth factors (NGF), insulin-like growth factors (IGF),** etc.
- Every growth factor has **its receptors** and binding of a growth factor with its cognate receptor causes conformational changes, leading to sending growth-promoting signals to the promoter of various genes inside the nucleus.
- Biochemically, both the growth factors and their receptors are proteins, so the interaction of growth factors and their receptors is said to be **protein-protein interaction**.
- Each growth factor acts on specialized cells. For example, VEGF activates the growth of vascular endothelial cells and vascular smooth muscle cells (Hornsby et al. 1983) (**see** Table 1).

5.2.9 Ethylene Diamine Tetraacetic Acid

- **Ethylene diamine tetraacetic acid** (**EDTA**) is a polyprotic acid containing four carboxylic groups and two amine groups with a lone pair of electrons that chelate calcium and several other metal ions.

Table 1 The table describes some common growth factors, their target cells, and the specific concentration needed for cell culture

Growth factor	Common arget cell	Specific target cell	Recommended concentration
EGF	Epiblasts, mesoblastoma	Keratinocytes, fibroblasts, chondrocytes, etc.	(1–2) $ng \cdot ml^{-1}$
FGF	Mesoblastoma, neuroectoderm	Fibroblasts and vascular cells	(0.5–10) $ng \cdot ml^{-1}$
IGF	Most cells	Most cells	(1–10) $ng \cdot ml^{-1}$
VEGF	Endothelial, smooth muscle, epithelial, etc.	Endothelial cells	(1–10) $ng \cdot ml^{-1}$
PDGF	Mesenchymal cells	Fibroblasts, myocytes, neurogliocytes	(1–50) $ng \cdot ml^{-1}$
NGF	Neuronal cells	Neurocyte, neuroglycocyte	(5–100) $ng \cdot ml^{-1}$

EGF = Epidermal Growth Factor, **FGF** = Fibroblast Growth Factor, **IGF**: Insulin-like Growth Factor, **VEGF** = Vascular Endothelial Growth Factor, **PDGF** = Platelet-Derived Growth Factor, **NGF** = Neuronal Growth Factor

- EDTA is described as a **metal chelator**, particularly for its strong reaction capacity with calcium and magnesium. Calcium and magnesium are necessary for cell attachment and therefore lead to cell aggregation and clumping following treatment with trypsin for cell harvesting from the culture containers. Thus, for cell harvesting from the culture medium, EDTA is always added to the culture medium.
- Figure 6 depicts the chemical structure of EDTA, wherein manifold OH groups near oxygen infer a significant role of intramolecular H-bonding. The disrupted nitrogen substituted aliphatic chains in the middle impart a distributed homogenization which helps in avoiding a sudden or random aggregation due to hydrophobic excess.
- EDTA is also used for **detaching adherent cells** from cell culture vessels either for passaging or just harvesting. The working concentration is 0.02%.
- Trypsin/EDTA treatment is a combined method for detaching cells. Of note, trypsin is a proteolytic enzyme. Generally, a mixture of 0.05% trypsin and 0.02% EDTA is directly added to the cell culture vessels for mammalian cell detachment.

5.2.10 Beta Mercaptoethanol

- **Beta mercaptoethanol (β-ME or 2-ME)** should be added afresh in some cell culture media.
- **β-ME or 2-ME is a reducing agent which breaks down disulfide** bonds and is added to the culture medium for T-cell proliferation. β-ME is supplied as 14.3 M (Fig. 7).
- The final concentration of β-ME in the culture medium is 50 μM.

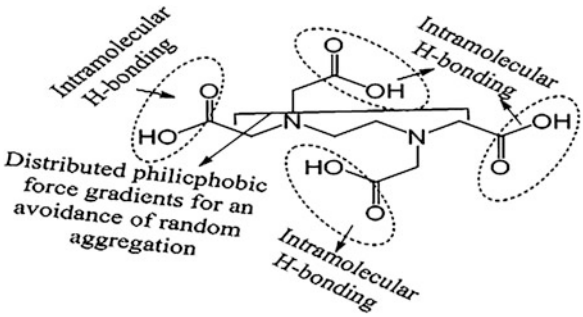

Fig. 6 Chemical structure of ethylene diamine tetraacetic acid (EDTA), with a distributive philicphobic force distribution-driven aggregation control and chelating ability

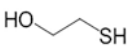

Fig. 7 Chemical structure of beta-mercaptoethanol (β-ME), with twin hydrogen donating sites, separated by an alkyl chain. Availability of H^+ confers reduction ability to this cell culture additive, which aids in T-cell proliferation

5.2.11 Serum

- ***The straw color serum is the blood plasma devoid of clotting factors***.
- The cell culture medium is supplemented with (5–10)% **Fetal Bovine Serum (FBS)** or **Fetal Calf Serum (FCS)** for promoting the healthy growth of mammalian cells.
- FBS/FCS has the highest quality because the fetal bovine is not exposed to the outside environment and has the lowest antibodies and complement.
- Before use, both FBS and FCS should be freed from the c**ompliment (destroying the serum complement proteins) by heating the serum at 56 °C for 30 min,** otherwise complements may lyse the cells in conjunction with antibodies.

NB: Complements are a series of around twenty heat-labile proteins secreted by the liver, and through a series of signaling cascades, enable antibodies and phagocytic cells to destroy or eliminate pathogens/antigens.

- The serum contains numerous hormones, growth factors, amino acids, peptides, and proteins (e.g., human serum albumin or HSA), glucose, fatty acids and glycerol, vitamins, minerals, enzymes, etc.
- Some specific culture conditions need specially treated serum. For example, when working on the effects of estrogens/other steroids on the in vitro cultured mammalian cells such as endothelial cells, ***charcoal-dextran treated serum*** is used. Charcoal-dextran **stripped all the steroids from the serum** and therefore effects of estrogens/other steroids on the endothelial cells would inevitably be prominent (von Seefried and Macmorine 1976; Hornsby et al. 1983).

5.3 Usefulness of Serum in the Cell Culture Medium

The addition of serum to the in vitro cultured mammalian cells has many beneficial effects including growth and proliferation, neutralization of trypsin and proteases that are used for the detachment of cells from the cell culture containers, neutralization of toxins that may be present in the cell culture medium, pH maintenance of the cell culture medium, and so on.

Here are some important beneficial effects of serum in cultured mammalian cells.

5.4 Essential Nutrients Are Provided by the Serum

While most mammalian cell culture medium contains 3–5% FCS/FBS, some cells need as high as 10% serum in the cell culture medium. Serum supplies all the essential nutrients including amino acids, vitamins, inorganic minerals, fats, and nucleic acid derivatives. Essentially, these materials help in cell growth and proliferation.

5.5 Adherence and Spreading of Cells Are Mediated by the Serum

As described previously, most mammalian cells are adherent in nature. So, these cells are not only cultured in polystyrene-made containers but also mandate the addition of cell-adhesive agents such as gelatin. The serum contains **fibronectin, laminin**, etc. which act as **adhesive agents** and therefore help in the cell adherence with the culture containers.

5.6 Hormones and Various Growth Factors Are Provided by the Serum

The serum contains various growth factors such as vascular endothelial growth factors (VEGFs), epidermal growth factors (EGFs), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), neuronal growth factors (NGOs), and others. The serum also contains both peptide/protein (e.g., insulin, glucagon, somatostatin, etc.) and steroid ((estradiol, testosterone, progesterone, etc.) hormones.

5.7 Serum-Mediated Protection for Some Specific Cells

The anti-protease ingredients present in the serum neutralize the various proteases released by the cells such as epithelial and myeloid cells.

Serum albumin maintains the viscosity of the mammalian cell culture medium and prevents mechanical damage to the cells.

Metabolic detoxification of the in vitro cultured cells is mediated by the trace elements and ions, such as SeO_3 and selenium.

The proteolytic enzymes such as trypsin facilitate the detachment of cultured adherent cells from the cultured containers, and harvesting of the cells is neutralized or diluted by the serum-containing used-up medium, before centrifugation of the cell suspension. This prevents trypsin-dependent damage to the plasma membrane of the mammalian cells.

5.8 Disadvantages of Using Serum in the Cell Culture Medium

- While there are several positive effects of adding a serum to a mammalian cell culture medium dedicated for in vitro mammalian cell culture, it has some negative effects also.
- Following digestion and absorption of food materials, all molecules enter the circulation through the intestine, from where they are once again transported into various cells. The intracellular composition and levels of these molecules differ from their plasma concentration, and unless there is an injury or damage to a cell, there is no direct contact of serum with intracellular compartments (exceptions

herein include the endothelial cells which are in direct contact with serum/blood). Thus, by adding a serum to the cell culture medium, the cultured cells are deliberately exposed to an artificial environment compared to physiological conditions.
- Serum composition may vary from batch to batch and, therefore, there may be a variation in results. This depends upon the nutrition and physiological status of the animals being used for the collection of serum.
- In highly proliferated cells, serum constituents such as spermine and spermidine can react with the polyamine to form toxic poly-spermine.
- In general, while serum is used for the growth promotion of mammalian cells (such as endothelial cells), it may inhibit the proliferation of a few cells such as **epidermal keratinocytes**.
- Serum may contain bacteriotoxin and antibodies against various pathogens that can affect cell growth or can even lead to cell death.
- Serum increases the difficulty of maintaining cultures during downstream processing.
- The serum is the most expensive component of a culture medium.
- Serum may be responsible for chance pathogenic contamination such as from mycoplasmas and some deadly human pathogenic viruses.

6 Classification of Media Based on the Presence or Absence of Serum

There are three types of media based on the presence or absence of serum in mammalian cell culture medium (Mather 1998).

They are as follows:

1. Basal medium
2. Reduced serum medium
3. Serum-free medium

One should choose the appropriate medium depending on the specific cell type to be cultured.

Ahead is a brief discussion of the three basic types of media.

6.1 Basal Medium

A basal medium is a defined medium containing essential and nonessential amino acids, vitamins, inorganic salts, organic compounds, and trace elements, but no other growth supplements are necessary for cell growth, for example, RPMI 1640 and DMEM with or without L-glutamine.

- Basal media without **Fetal Bovine Serum (FBS)** or **Fetal Calf Serum (FCS)** is called an **incomplete medium**.

- For providing full growth of cells, **10% decomplemented** and **endotoxin-free serum** is always added to all basal media like RPMI-1640 and DMEM prior experiment.
- All culture media supplemented with 10% decomplemented FBS or FCS is referred to as **complete media** (Hornsby et al. 1983).

6.2 Reduced Serum Medium

- **Reduced Serum Medium** is the basal medium enriched with nutrient-derived factors, which reduce the needed serum content.
- Normally, basal mediums with **(2–5)% serum** are called **reduced serum medium**.

6.3 Serum-Free Medium

- **Basal medium** with lots of additional supplements in absence of any serum is called **serum-free medium**.
- Several published papers and books are available describing the process of development of serum-free medium (Hewlett 1991; Karmiol 2000).
- The major benefit of using a **serum-free medium** includes the flexibility to choose a medium in absence of serum, depending on different nutrients and growth factors for specific cell types.
- Thus, a **serum-free medium** replaces the necessity of adding animal serum with appropriate nutritional and hormonal formulations.
- In the case of many primary cultures and cell lines like Chinese Hamster Ovary (**CHO**), hybridoma cell lines, **VERO**, and **MDCK**, a **serum-free medium** is used.
- The **basal medium** contains **vitamins, amino acids, nucleic acids, lipids, inorganic salts**, and an **energy source**.
- Additional supplements in a **serum-free medium** include growth factors such as **insulin-like growth factors** and **epidermal growth factors**.
- **Attachment factors present in serum-free media include collagen** and **fibronectin**, **transport proteins**, **detoxifying agents** like **albumin** and **transferrin, trace elements** (Fe, Cu, Sn, Co, Mn), and **lipids** like **essential fatty acids** and **phospholipids and various hormones, etc**. (Iscove and Melchers 1978; Bottenstein et al. 1979; Barnes and Sato 1979, 1980; Brunner et al. 2010).
- **Hematopoietic cells** were cultured in a serum-free medium supplemented with selenite, transferrin, albumin, and lecithin (Guilbert and Iscove 1976).

6.4 Advantages of Using A Serum-Free Medium

- The major use of the serum-free medium is bioprocessing industries for the production of various products in a cost-savings way (cheaper).
- The serum is the source of various microbial contaminations in the cell culture medium including mycoplasma. By eliminating the use of serum in the cell culture medium, the chances of microbial contamination will be reduced.
- One of the major products of bioprocessing industries is the production of various proteins that are utilized for different purposes including the production of antibodies, vaccines, industrial utilization as enzymes, or even therapeutic use. The serum contains a large number of proteins which interferes with the purification of the protein of interest. Therefore, in this case, the absence of serum is highly preferred for easier purification and downstream processing.
- In absence of serum constituents, it will be easier to control the physiological responsiveness of the cultured cells in response to various types of drugs/toxins or other molecules.
- Following the experimental observation, the evaluation of data is very precise and consistent.
- As mentioned above, serum-free media are widely utilized for the production of various recombinant proteins by culturing various mammalian cells such as CHO cells (Keen and Rapson 1995).
- Protein-free media are also utilized to culture CHO cells (Hamilton and Ham 1977).
- Also, hybridoma cells that are cultured for monoclonal antibody production use a serum-free medium (Murakami et al. 1982; Murakami 1989).

6.5 Cautions to be Exercised in a Serum-Free Culture

Overall, cells in serum-free culture are more sensitive to extremes of pH, temperature, osmolality, mechanical forces, and enzyme treatment. Several precautionary steps must be needed not only to protect the cultured cells in a serum-free medium but also to protect the cell culture products (Merten 1999).

The following precautionary measures should be followed for serum-free culture of the mammalian cells.

6.6 Regarding the Use of Antibiotics and Antimycotics in Serum-Free Medium

Antibiotics are substances produced by one group of microorganisms and act against another group of microorganisms in a low concentration**.** On the other hand, antimycotics acts against fungi. In general, a serum-containing medium that is used in the research laboratories used antibiotics such as penicillin/ampicillin and

antimycotics such as amphotericin B to prevent the contamination of microorganisms during the mammalian cell handling and culture.

For mammalian cell culture in serum-free medium, either no antibiotics/antimycotics should be used or if one intends to use antibiotics and antimycotics in the cell culture medium, these must be used at 5–10 times lower concentration. This is because, in serum-containing media, various proteins present in the cell culture medium interact with antibiotics and therefore reduce the availability of free antibiotics and antimycotics. Without serum, the entire concentration of antibiotics and antimycotics remains unbound and therefore may directly act on mammalian cells and damage them.

6.7 Regarding the Maintenance of Cell Density in Serum-Free Medium

During subculturing or passaging of cells, a fixed proportion of cells always perishes due to various reasons. So, seeding at a higher density is always helpful to achieve the required cell population in a fixed time.

- Another reason for maintaining the seeding density is maintaining the cell-to-cell cross-talk or communication via secreting various materials. Cells may not grow properly due to inadequate cell-to-cell communication. For example, it has been observed that experimentally, endothelial cells fail to grow if the seeding density is less than 1 x 10^5 in a 100 mm Petri plate.
- It is recommended that mid-log phase cells having confluency of ~60–80% should be passed. In general, 100% confluent cells (already subject to contact inhibition) take a longer time to adapt and grow when passaged into new cell culture containers.
- Adaptation to a serum-free medium should be slow, gradual, and progressive. Sudden changes from the serum-containing to the serum-free medium may generate shock, resulting in either the cell's failure to respond to the culture medium for growth or even death.

6.8 Morphological Changes During Shifting of Cells from Serum-Containing to Serum-Free Medium

During the transfer of cells from serum-containing to serum-free medium, there may be a slight change in the morphology of the cultured cells. In general, such morphological changes do not exhibit effects on the overall properties of the cultured cells. However, if the doubling time and viability of the cells alter, caution should be taken and the situation demands a change in the culture protocol.

6.9 Clumping During Shifting of Cells from Serum-Containing to Serum-Free Medium

As one gradually adapts cells from serum-containing to serum-free medium, there may be clumping of the cells. In this condition, it is recommended to gently or slowly triturate the clumps to break them before passaging of cells.

6.10 Formulation of Serum-Free Medium

To prepare a serum-free medium, several supplements are added to the basic cell culture medium.

The following are the list of supplements.

6.10.1 Adherence Substances

Previous sections of this chapter discussed in detail the adherent cells and the adherent substances such as laminin, fibronectin needed for the mammalian cell culture.

6.10.2 Growth Factors

Growth factors are needed for the optimum growth of various cells. For example, VEGF is necessary for the culture of endothelial cells. This point is discussed in the earlier section of this chapter.

6.10.3 Peptide or Steroid Hormones

Depending upon the type of cells, either peptide or steroid hormones may need to be added to the culture medium.

6.10.4 Enzyme Inhibitors

Adherent cells require trypsinization to passage. Trypsin is a proteolytic enzyme and therefore can damage or injure the plasma membrane if it is not neutralized following detachment of adherent cells from the culture containers. In such an eventuality, a **soybean trypsin inhibitor** may be used to neutralize trypsin.

6.10.5 Binding Protein(S) and Translocator

In the earlier section of this chapter, it has been mentioned that several translocators and binding proteins are required for the mammalian cell culture. The specific type and amount depend on the cells. For example, bovine serum albumin (**BSA**) and **transferrin** are required by some cells.

6.10.6 Trace Elements

In the earlier section of this chapter, it has been mentioned that several trace elements are required for the mammalian cell culture. For example, **selenium** is required by some cultured cells.

6.10.7 Process of Using Serum-Free Medium

In general, cells are grown in a serum-containing medium after which, the serum is gradually removed from the culture medium so that cells should not witness a sudden nutritional crunch.

The process of gradual adaptation is as follows:

- Grow cells in a 10% serum-containing medium.
- Subculture the cells in a 5% serum-containing medium.
- Subculture the cells in a 3% serum-containing medium.
- Subculture the cells in a 1% serum-containing medium.
- After 70–90% confluency, remove serum from the culture containers and allow the cells to adapt to a serum-free medium.

6.11 Protein-Free and Chemically Defined Media

- A chemically defined medium (**CDM**) is a medium in which the chemical nature and extents of all constituents are known.
- Serum and protein-free media may be highly useful to produce recombinant proteins using mammalian cell culture.
- In the bioprocessing industry, Chinese Hamster Ovary (**CHO**) cells are highly used for the production of various recombinant proteins. The CHO cells are adapted to grow in a protein-free medium.
- At present, several companies use the protein-free medium for the culture of mammalian cells to produce various products.

6.12 Types of Culture Media

Mammalian cell culture media can be classified as follows (Darling and Morgan 1994; Macy and Shannon 1977).

Natural Medium
Synthetic Medium

The following paragraphs describe the basics of these media configurations.

6.12.1 Natural Medium

- **Animal body fluid** (**e.g.,** plasma, serum, lymph) **or tissue extraction** (chicken embryos leaching solution) is recognized as a natural medium.
- While natural media are rich in every type of nutrition required for the culture of mammalian cells, there are batch-to-batch variations in the composition of this type of medium.
- Therefore, these types of media are replaced by synthetic medium with a standardized/fixed concentration of various nutrients.

6.12.2 Synthetic Medium

Commercial culture media were developed as early as the 1950s. The first-ever developed medium was **Eagle's Basal Medium** by Harry Eagle during normal and malignant cell type studies. **Eagle's Basal Medium** was later modified to generate many popular media (Jacoby and Darke 1948; Ham 1965; Ham and McKeehan 1979).

Here is a brief description of some common mammalian cell culture media.

7 Common Cell Culture Media

Minimum Essential Medium (**MEM**)
Alpha Minimum Essential Medium (**α-MEM**)
Glasgow Minimum Essential Medium (**GMEM**)
Dulbecco's Modified Eagle's Medium (**DMEM**)
Iscove's Modified Dulbecco's Medium (**IMDM**)
Roswell Park Memorial Institute Medium (**RPMI**)
199medium (**M199 MEDIUM**)
Ham's F10 Medium
Ham's F12 Medium
Kaighn Modified Ham's F-12 Medium (**Ham's F-12 k**)
Dmem-F12 Medium
RPMI 1640/DMEM/F-12 (**RDF**) Medium
McCoy's 5A Medium
Leibovitz L15 Medium
Waymouth's MB752/1 Medium
Trowell's T8 Medium

Each medium has its composition and supplements (Freshney 2010).
Ahead is a brief description of the above cell culture media configurations.

7.1 Minimum Essential Medium

- Minimum Essential Medium (**MEM**), also called **Eagle's Minimal Essential Medium (EMEM)**, is the cell culture medium developed by ***Harry Eagle***.
- Eagle first developed Basal Medium Eagle (**BME**), and then gradually increased the content of essential amino acids to produce **MEM** (Table 2).
- However, it only contains 12 kinds of nonessential amino acids, glutamine, eight vitamins, and some basic inorganic salts.
- Many continuous mammalian cell lines such as MCF-7 and MDA-MB breast cancer cells and adherent cells are maintained in this simple medium.

Table 2 Basic differences between the Basal Medium Eagle (**BME**) and Minimal Essential Medium (**MEM**) compositions

Medium	BME	MEM
Inorganic salts		
Calcium chloride ($CaCl_2$) (anhydrous)	200	200
Magnesium sulfate ($MgSO_4$) (anhydrous)	97.67	97.67
Potassium chloride (KCl)	400	400
Sodium bicarbonate ($NaHCO_3$)	2200	2200
Sodium chloride (NaCl)	6800	6800
Sodium phosphate monobasic (NaH_2PO_4-H_2O)	140	140
Vitamins	1	
Biotin		
Choline chloride	1	1
D-calcium pantothenate	1	1
Folic acid	1	1
Niacinamide	1	1
Pyridoxal hydrochloride	1	1
Riboflavin	0.1	0.1
Thiamine hydrochloride	1	1
i-inositol	2	2
Amino acids		
L-arginine hydrochloride	21	126
L-Cystine 2HCl	16	31
L-glutamine	292	292
L-histidine	8	
L-histidine hydrochloride-H_2O		42
L-isoleucine	26	52
L-leucine	26	52
L-lysine hydrochloride	36.47	73
L-methionine	7.5	15
L-phenylalanine	16.5	32
L-threonine	24	48
L-tryptophan	4	10
L-tyrosine disodium salt dehydrates	26	52
L-valine	23.5	46
Other materials		
D-glucose (dextrose)	1000	1000
Phenol red	10	10

7.2 Alpha Minimum Essential Medium

- Minimum Essential Medium Eagle-alpha modification is a medium based on MEM, first published in 1971 by ***Clifford P. Stanners*** **and colleagues**.

- It contains significant nonessential amino acids, sodium pyruvate, and vitamins (ascorbic acid (vitamin C), biotin, and cyanocobalamin) compared with MEM (Table 3).
- It is also available with lipoic acid and nucleosides.

NB: Some researchers may prefer to add nucleosides as well as fresh L-glutamine, in the culture media.

7.3 Glasgow Minimum Essential Medium

Ian MacPherson and Michael Stoker modified Eagle's medium and named it **Glasgow** Modified Essential Medium **(GMEM)**.

The modification includes the addition of 10% tryptose phosphate and twice the normal concentration of amino acids and vitamins.

Table 3 Composition of Alpha-Minimum Essential Medium

Components	Concentration (mg/L)
Inorganic salts	
$CaCl_2$ (anhydrous)	200.00
KCl	400.00
$MgSO_4$ (anhydrous)	97.67
NaCl	6800.00
$NaHCO_3$	2200.00
$NaH_2PO_4 \cdot H_2O$	140.00
Other components	
D-glucose	1000.00
Lipoic acid	0.20
Phenol red	10.00
Sodium pyruvate	110.00
Amino acids	
L-alanine	25.00
L-arginine	105.00
L-asparagine-H_2O	50.00
L-aspartic acid	30.00
L-Cystine	24.00
L-cysteine-HCl-H_2O	100.00
L-glutamic acid	75.00
L-glutamine	292.00
Glycine	50.00
L-histidine	31.00
L-isoleucine	52.50
L-leucine	52.40
L-lysine	58.00

(continued)

Table 3 (continued)

Components	Concentration (mg/L)
L-methionine	15.00
L-phenylalanine	32.00
L-Proline	40.00
L-serine	25.00
L-threonine	48.00
L-tryptophan	10.00
L-tyrosine	36.00
L-valine	46.00
Vitamins	
L-ascorbic acid	50.00
Biotin	0.10
D-ca Pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-inositol	2.00
Niacinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00
Vitamin B12	1.36

- This medium is specially designed to grow certain cells.
- Particularly, this medium was initially designed to grow the Baby Hamster Kidney cells **(BHK-21)** to examine the genetic factors impacting cell competence.

NB: The medium composition/formula is available from the websites of various companies.

7.4 Dulbecco's Modified Eagle's Medium

- ***Dulbecco/Vogt*** modified Eagle's Minimal Essential Medium **(MEM)** and renamed it **Dulbecco's Modified Eagle's Medium (DMEM)**.
- The modification in DMEM includes the addition of ferric nitrate (**iron**), phenol red **(to check the pH alteration)**, fourfold vitamins, and twofold amino acids over the MEM.
- In addition, based on the cell's need (e.g., some tumor cells need a high concentration of glucose) of carbon source, either a **low (1000 g•L^{-1})** or a **high (4500 g•L^{-1})** glucose is used. So, DMEM is divided into low and high glucose regimes.

- Since the medium is very rich in vitamins, amino acids, glucose, etc., most of the cells grow easily in this type of medium.
- However, cells requiring special medium composition should grow in that specific medium composition.
- For example, to show the effects of estrogens (a female hormone) on the endothelial cells nitric oxide (NO), growth factors, hormones, phenol red, etc. must be removed from the cells.

NB: The medium composition/formula is available on the websites of various companies.

7.5 Iscove's Modified Dulbecco's Medium

- Iscove added 12 ingredients including amino acids and vitamins, sodium pyruvate, selenium, potassium nitrate, and HEPES in DMEM and renamed it ***Iscove's*** Modified Dulbecco's Medium **(IMDM)**.
- Iron (ferric nitrate) is removed from the medium.
- Iscove claimed that this modified medium may be suitable for the growth of transformed cells after DNA transfection, low-density cell cultures, and difficult cell cultures.
- IMDM is also used as growth support for hematopoietic precursor cells and various lymphocytes.

NB: The medium composition/formula is available from the websites of various companies.

7.6 Roswell Park Memorial Institute Medium

- In 1966, ***Moore and his group*** at Roswell Park Memorial Institute, USA, developed a new medium, which was named Roswell Park Memorial Institute Medium (**RPMI**).
- The medium was further modified and renamed **RPMI-1640.** It is one of the most commonly used media, particularly useful for suspension cells, mainly **lymphoid cells**.
- However, many other types of cells including primary cells can easily grow in this medium.
- One liter of RPMI-1640 contains the following: glucose (2 g) pH indicator (phenol red, 5 mg), salts (6 g sodium chloride, 2 g sodium bicarbonate, 1.512 g disodium phosphate, 400 mg potassium chloride, 100 mg magnesium sulfate and 100 mg calcium nitrate) (Table 4).

NB: The medium composition/formula is available from the websites of various companies.

Table 4 Composition of the RPMI medium

Roswell Park memorial institute (RPMI) media	RPMI 1640 (Moore and Gerner 1967)	This medium is originally formulated based on the 5A Medium which was developed by McCoy et al. in 1959. The purpose to develop this medium was the long-term culture of peripheral blood lymphocytes and hybridoma cells characterized by low levels of calcium and magnesium and high levels of phosphate. Later on, the medium composition was modified several times and the media were renamed (e.g. RPMI 1629, 1630, and 1634).

7.7 M 199 Medium

- In 1950, ***Morgan, Morton, and Parker*** for the first time developed a nutritionally defined medium for cell culture. Thus, it is one of the earliest culture media. The medium was named "medium 199 or M-199."
- Composition analysis of M-199 shows more than 60 distinct components including almost all amino acids, vitamins, growth hormones, nucleic acid derivatives, etc., to varying extents.
- HBSS or ESS was used as a basic solvent to prepare this medium.
- AT094A is M199 with Earle's salts and 25 mM HEPES buffer. It does not contain L-Glutamine.
- Originally, this medium was developed to culture fibroblasts from chick embryos.
- It was observed that explanted tissues could survive in Medium 199 without serum but long-term cultivation of cells required supplementation with serum.
- A large number of cells including various endothelial and epithelial cells, transformed cells for vaccine and virus production, and primary explants culture can be grown in M199 with serum supplementation.

NB: The medium composition/formula can be obtained from the websites of various companies.

7.8 HAM'S F-10 Medium

- Developed in 1962–1963 by ***Ham***.
- This medium was originally designed in 1962, to support the growth of mouse and human diploid cells.
- Originally, no serum was added to the medium, but subsequently, albumin and fetuin were added as serum constituents.
- Trace elements, copper, and zinc were added for the first time in this culture medium.

- It was observed that CHO cells grew well in this medium without serum supplementation. This was a major boon for biopharmaceutical and bioprocessing industries to produce various cell-based compounds.
- However, culturing other cells in this medium may require serum supplementation.

NB: The medium composition/formula is available from various company websites.

7.9 Ham's F-12 Medium

- In 1965, ***Ham*** developed this medium.
- This medium is claimed to be the world's first chemically defined medium.
- The basic distinctions between Ham's F10 and Ham's F12 media comprise the serum albumin and fetuin replacements with linoleic acid and putrescence, respectively.
- Most of the amino acids are present to a greater extent in Ham's F12 medium than in Ham's F10 medium while those of the vitamins (except choline and inositol) and potassium phosphate are lower in Ham's F12 medium.
- The concentration of zinc is also reduced for a protein-free culture of mammalian cells such as the CHO cells.
- This medium is also available with a 25 mM HEPES buffer, yielding a more effective buffering within the 7.2–7.4 optimum pH range.
- MCDB301 was later developed with 20 trace elements for a protein-free culture of CHO cells in Ham's F-12 medium.
- Some cell types cultured in this medium are primary rat hepatocytes, rat prostate epithelial cells, and CHO cells, without any serum requirement.

NB: The medium composition/formula is available on the websites of various companies.

7.10 Kaighn Modified Ham's F-12 Medium

- In 1974, ***Kaighn*** modified Ham's F12 medium, thereby renaming it Ham's F-12 K culture medium.
- This modification was aimed at the primary culture of mammalian cells.
- Concerning Ham's F-12, the concentrations of the amino acids, pyruvate, biotin, calcium, magnesium, putrescence, and phenol red were higher in this culture medium.

NB: The medium composition/formula is available on the websites of various companies.

7.11 DMEM-F12 Medium

- This medium is a **1:1** mixture of Ham's **F-12** and **DMEM**, combining the richness of F12 with DMEM nutritive potency.
- It is widely used for cell culture in serum-free conditions.
- In 1979, ***Barnes and Sato*** formulated a 50:50 mixture of nutrient-rich DMEM medium and Ham's F12 medium. This medium mixture was subsequently recommended as the basal medium for the serum-free culture of several mammalian cells.

NB: The medium composition/formula is available on the websites of various companies.

7.12 RPMI-1640/DMEM/F-12 Medium

- In 1984, a new cell culture medium was developed by ***Murakami*** for the serum-free culture of hybridomas.
- It is typically used as a serum-free mode or after being supplemented with insulin, transferrin, ethanolamine, and selenite.
- This medium was developed in a 2:1:1 mixture of RPMI 1640, DMEM, and Ham's F-12, resulting in the terminology of **RDF medium**.

NB: The medium composition/formula is available on the websites of various companies.

7.13 McCoy's 5A Medium

- In 1959, ***McCoy*** and his colleagues reported the amino acid requirements for in vitro cultivation of ***Novikoff Hepatoma Cells***.
- These studies were performed using Basal Medium 5A, resulting in subsequent modification and creation of a new medium known as McCoy's 5A Medium.
- At present, McCoy's 5 M medium is used for the culture of several primary as well as established cell lines.
- The primary cultures derived from adrenal glands, bone marrow (normal), gingiva, lung, mouse kidney, skin, spleen, and other tissues use this culture medium.

NB: The medium composition/formula is available on the websites of various companies.

7.14 Leibovitz L15 Medium

- In 1963, ***Leibovitz*** developed the L15 medium.
- The buffering capacities of this medium are attributed to phosphates and free basic amino acids instead of sodium bicarbonate so that the culture's pH is maintained in ambient air without a CO_2 incubator.
- Amino acids were added to this medium at their highest concentration.
- Instead of glucose, pyruvate (and galactose) is added at a high concentration to control pH decline due to lactic acid production.
- At present this medium is rarely used.

NB: The medium composition/formula is available on the websites of various companies.

7.15 Waymouth's Mb752/1 Medium (Waymouth 1959)

- In 1959, ***Waymouth*** developed this medium.
- This medium was developed with a simple composition as feasible, so that **mouse L929 cells** could be cultured without adding a serum and other proteins.
- This medium is composed of a total of 40 components, including glucose, inorganic salts, amino acids, vitamins, purine bases, and hypoxanthine.
- It is characterized by high concentrations of glucose, histidine, lysine, glutamine, choline, and thiamine.

NB: The medium composition/formula is available from the websites of various companies.

7.16 Trowell's T-8 Medium

- In 1959, ***Trowell*** developed this medium.
- This medium is suited for the long-term culture of **adult rat liver epithelial cells**.
- With its reasonably simple composition, this medium is devoid of any nonessential amino acids as well as vitamins but rather has high glucose and insulin contents.
- It is used for short-term organ culture.

7.16.1 General Observations Regarding Mammalian Cell Culture Media

- The above-stated media are well-commercialized with each having different physical or packaged forms, such as powder or liquid, large or pouch pack.
- The liquid form is subdivided into 10x concentrated solution, 2x concentrated solution, and working solution (1x).
- Ca^{+2} and Mg^{+2} are missing in some culture media.

- Phenol red (pH indicator of cell culture medium) is absent in some culture media since it may interfere with a certain cell culture treatment.
- Glucose may be added to the media in low, medium, or high concentrations.
- The laboratory personnel/users can choose the product according to experimental requirement(s), particularly the type(s) of cells used.

8 Medium Recommendations for Common Cell Lines

- In the research laboratories or the research and development section in a pharmaceutical company/bioprocessing industry, many continuous mammalian cell lines can be maintained on a relatively simple medium such as **MEM** supplemented with serum.
- However, as mentioned in the previous section, large-scale culture in the bioprocessing industry requires serum-free and protein-free media, for cost-saving and other reasons.
- For desired specialized cells, a specialized medium must be used for optimum growth.
- Since the information for selecting the appropriate medium for a given cell type is usually available in published literature, the responsible person must read and survey the literature. For example, cells available from American Type Culture Collection **(ATCC)** carry all the details of a cell culture medium and its composition.
- If there is no information available about a specific medium for a specific cell type, one can choose the growth medium and serum empirically or test using several different media for best results.
- It is generally recommended that a good way to start mammalian cell culture is by using ***MEM for adherent cells and RPMI-1640 for suspension cells***.
- In general, for large-scale culture of mammalian cells in a bioreactor or similar containers for the production of specific cell culture products, a serum-free medium is preferable.
- Serum-free medium is not only cost-effective but also decreases the contamination of deadly microbes or viruses through serum contamination (Eisenblatter et al. 2002).

Table 5 ***describes some recommended media for the culture of common mammalian cells.***

8.1 Recommended Media for Common Cell Types

The following table describes various cell lines isolated from the *Homo sapiens* (humans).

Table 5 Recommended medium for the culture of common mammalian cells

Cell line	Cell type	Species	Tissue	Medium
HUVECS	Endothelial (primary cell)	Human	Umbilical cord	F-12 K and 10% FBS plus 100 μg/ml heparin or M199 with 5% serum VEGF and heparin
HPAECs	Endothelial (primary cell)	Human	Human pulmonary artery	M199 with 5% serum, VEGF, heparin
HMEC-1	Endothelial Cell line	Human	Dermal microvessels	M199 with 5% serum, VEGF, heparin
MCF-7	Epithelial cell line	Human	Breast adenocarcinoma	MEM and 5–10% FBS plus NEAA and 10 $\mu g.ml^{-1}$ insulin DMEM can also be used instead of MEM
MDAMB-468	Epithelial Cell line	Human	Metastatic breast adenocarcinoma	ATCC-formulated Leibovitz's L-15 medium, with 10%FBS, cells also grow well in MEM/DMEM and (5–10)% FBS plus NEAA and 10 μg/ml insulin.
A549	Epithelial cell line	Human	Lung carcinoma	Cells grow well in F-12 K medium (with 10% FBS and penicillin (100 $U{\cdot}ml^{-1}$), streptomycin 100 $\mu g{\cdot}ml^{-1}$). Cells also grow well in DMEM with high glucose (4.5 to 5 gm glucose/liter of medium).
BEAS-2B	Epithelial cell line	Human	Bronchus of lung	BEGM, also known as LHC-9 with modification or BEBM bullet kit (available from Clonetics) is claimed to be the best culture medium.
HeLa	Epithelial Cell line	Human	Cervical carcinoma	MEM and 10% FBS plus NEAA (in suspension, S-MEM)
LNCaP	Epithelial cells	Human	Male prostate adenocarcinoma	RPMI-1640 with 10%FBS, 100 U/ml penicillin, 100 μM streptomycin sulfate
PC3	Epithelial cell	Human	Male prostate neuroendocrine carcinoma	F-12 K with 10% FBS, 100 U/ml penicillin, 100 μM streptomycin sulfate
HT-1080	Epithelial Cell line	Human	Fibrosarcoma	MEM and 10% HI FBS plus NEAA
Caco-2	Epithelial Cell line	Human	Colon adenocarcinoma	MEM and 20% FBS plus NEAA
VERO	Epithelial cell line	African green monkey	Kidney cancer	EMEM (ATCC formulated) with 10%FBS, or DMEM with high glucose.
HEK-293	Fibroblast Cell line	Humans	Embryonic kidney	MEM and 10% FBS

(continued)

Table 5 (continued)

Cell line	Cell type	Species	Tissue	Medium
COS-7	Fibroblast Cell line	Monkey	Kidney	DMEM and 10% FBS
RAW	Macrophage cell line	Abelson mouse	Blood cell (macrophage) Abelson murine leukemia virus-induced tumor, ascites	DMEM with high glucose, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate.
Jurkat	Lymphoblast cell line	Human	Lymphoma (T cell)	RPMI-1640 and 10% FBS

HUVECs: Human Umbilical Vein Endothelial Cells; **HPAECs:** Human Pulmonary Artery Endothelial Cells; **HMEC-1:** Human Microvascular Endothelial Cells-1; **MCF-7:**Michigan Cancer **Foundation**-7**; MDAMB-468:** MDA stands for "M.D. Anderson, MB stands for Metastatic Breast Cancer" and 468 is an abrupt number; **HeLa: Henrietta Lacks; HEK-293:**Human Embryonic Kidney 293; **Caco-2:**Cancer coli-2; **COS-7: C**V-1 (Simian) in **O**rigin, and carrying the **S**V40 genetic material; **MEM:** Minimum Essential Medium; **DMEM:** Dulbecco's Modified Eagle Medium; **NEAA:** Nonessential Amino Acids Solution, **F12K:** Kaighn's modification of Ham's K12 medium; **FBS:** Fetal Bovine Serum

9 Common Buffer and Solutions for Cell Culture

In the absence of a culture medium, **Balanced Salt Solution (BSS)** can serve as an in vitro microenvironment for cells for a short period that helps to maintain the structural and physiological integrity of the cells. They also maintain **pH**, osmotic pressure (**osmolality**), and m**embrane potential** ($Na^{+,}$ $K^{+,}$ Ca^{+2}) besides providing i**ons** for a**ttachment** and binding of e**nzyme cofactors** (Eagle 1956; Eagle 1971).

9.1 Mechanism of Action of Various Basal Salt Solutions

- Na^{+} and K^{+} maintain an isotonic solution, resulting in optimum osmolarity, and permeability of mammalian cells.
- Ca^{+2} and Mg^{+2} maintain the internal structure of the cells as well as the integrity of the plasma membrane.
- Phosphate and bicarbonate are the buffering agents. They maintain the H^{+} concentration of the solutions.
- Glucose or pyruvate acts as a possible energy source.

Some regularly used BSS for keeping cells at room temperature are as follows:

1. Earle's balanced salt solution
2. Hanks balanced salt solution
3. Phosphate buffer saline

4. Dulbecco's phosphate buffer saline with or without Ca^{++} and Mg^{++}
5. Normal saline
6. Alsever's solution

Here is the description of the process of preparation of the above buffers/ solutions.

9.2 Preparation of Earle's Balanced Salt Solution

- **Earle's** Balanced **Salt** Solution (**EBSS**) is an isotonic buffer solution containing inorganic salts such as sodium chloride (NaCl), potassium chloride (KCl), and carbohydrate as an energy source, bicarbonate and phosphate for buffering agents (Table 6).
- EBSS is manufactured with and without phenol red and Ca^{+2} and Mg^{+2}.
- This buffer is designed for the short-term maintenance of cells in a CO_2 environment.
- EBSS is commonly used for different cell culture applications such as washing, transporting, and diluting the cells.

NB: Added phenol red content maybe 10 mg per liter.

9.2.1 Procedure for Making Earle's Balanced Salt Solution (1 Liter)

1. Take 800 ml distilled water in a suitable container.
2. Add 200 mg $CaCl_2$ (anhydrous) to the solution.
3. Add 200 mg $MgSO_4$ (heptahydrate) to the solution.
4. Add 400 mg KCl to the solution.
5. Add 2.2 gm $NaHCO_3$ to the solution.
6. Add 6.8 gm NaCl to the solution.
7. Add 140 mg Na_2HPO_4 (monobasic) to the solution.
8. Add 1gm glucose or dextrose to the solution.
9. Add 10 mg phenol red to the solution.
10. Adjust solution to final desired pH using HCl or NaOH.
11. Add distilled water until the final volume is adjusted to 1 liter.

Table 6 Composition of **Earle's** Balanced **Salt** Solution (**EBSS**)

Name	Concentration (M)	The amount for 1 liter (mg)
$CaCl_2$	0.001	200
$MgCl_2$	0.0008	200
KCl	0.005	400
$NaHCO_3$	0.026	2200
NaCl	0.117	6800
Na_2HPO_4	0.001	140
D glucose	0.006	1000

9.2.2 Preparation of Hank's Balanced Salt Solution

- **Hanks' Balanced Salt Solution (HBSS)** consists of inorganic **salts including phosphate** apart from being supplemented with glucose and used to maintain a physiological pH and osmotic pressure of the cultured mammalian cells (Table 7).
- It is also used to wash cells besides maintaining them in a viable state.
- Additionally, HBSS provides water and essential inorganic ions to cells.
- HBSS is available in multiple formats including Ca and Mg-free versions for washing cells before trypsinization.
- Phenol red can also be added to HBSS.

9.2.3 Procedure for Making Hanks Balanced Salt Solution

1. Take 800 ml distilled water in a suitable container.
2. Add 8 gm NaCl to 800 ml distilled water.
3. Add 400 mg KCl to the solution.
4. Add 140 mg $CaCl_2$ to the solution.
5. Add 100 mg $MgSO_4 \cdot 7H_2O$ to the solution.
6. Add 100 mg $MgCl_2 \cdot 6H_2O$ to the solution.
7. Add 60 mg Na_2HPO_4-$2H_2O$ to the solution.
8. Add 60 mg of KH_2PO_4 to the solution.
9. Add 1 gm glucose to the solution.
10. Add 350 mg $NaHCO_3$ to the solution.
11. Add distilled water until the final volume is 1 liter.

9.2.4 Preparation of Phosphate Buffer Saline

- **Phosphate-buffered saline** (**PBS**) is a buffer solution commonly used in biological research.
- It is a water-based salt solution containing Na_2HPO_4 **disodium hydrogen phosphate**, NaCl, and in some formulations, KCl and KH_2PO_4 (Table 8).
- It closely mimics the pH, osmolarity, and ion concentrations of the human body.
- The buffer helps to maintain a constant pH.
- PBS has many uses because it is isotonic and nontoxic to cells.

Table 7 Composition of Hanks' Balanced Salt Solution (**HBSS)**

Name	Concentration (M)	Amount per 1 liter (mg)
NaCl	0.14	8000
KCl	0.005	400
$CaCl_2$	0.001	140
$MgSO_4$	0.0004	100
$MgCl_2$	0.0005	100
Na_2HPO_4	0.0003	60
KH_2PO_4	0.0004	60
Glucose	0.006	1000
$NaHCO_3$	0.004	350

- The major use of sterile PBS buffer in a mammalian cell culture laboratory is for the aseptic washing of cells/tissue, washing of cell culture containers, following adhesive molecule treatment, washing of the cells following trypsinization, dilution of medium constituents and water-soluble drugs, toxins, etc.

9.2.5 Procedure for Making Phosphate Buffer Saline

Take 800 ml distilled water in a suitable container.
Add NaCl, KCl, KH_2PO_4, and $Na_2HPO_4.2H_2O$.

The final pH is adjusted between (7.2–7.4) using NaOH or HCl.

Adjust final volume to 1 liter using double-distilled H_2O, autoclave, and then store.

NB: PBS is the cheapest, most widely used buffer during cell culture including isolation of cells and posttreatment (with the drug of interest) washing.

9.3 Preparation of Dulbecco's Phosphate Buffer Saline

- ***Dulbecco*** is the name of a scientist who modified the composition of the PBS that suits some mammalian cells washing in a PBS. It is named after the discoverer.
- **Dulbecco's Phosphate Buffered Saline** (**DPBS**) is intended to provide a **buffer** system for maintaining cell culture medium in the physiological **pH** range of 7.2–7.6.
- It provides cells with water and certain bulk inorganic ions essential for normal cell metabolism. Table 9 contains the Ca^{+2} and Mg^{+2} devoid of DPBS constituents.

NB: The presence of Ca^{+2} and Mg^{+2} promote adhesions. So, the removal of Ca^{++} and Mg^{++} helps in cell detachment.

Table 8 Composition of phosphate-buffered saline (**PBS**)

Name	Concentration	Amount (in g) per liter
NaCl	0.137 M	8
KCl	0.0027 M	0.2
Na_2HPO_4	0.01 M	1.44
KH_2PO_4	0.0018 M	0.240

Table 9 Composition of Dulbecco's Phosphate Buffered Saline (**DPBS**) without calcium and magnesium

Name	Concentration (mM)	Amount (in gm) for 1 liter
NaCl	136.9	8
KCl	2.67	0.2
KH_2PO_4	1.47	0.2
Na_2HPO_4, anhydrous	8.10	1.15

9.3.1 Procedure for Making Dulbecco's Phosphate Buffer Saline Without Ca^{+2} and Mg^{+2}

Take 800 ml distilled water in a suitable container.
Add NaCl, KCl, KH_2PO_4, and $Na_2HPO_4 \cdot 2H_2O$.
Adjust final pH within (7.2–7.4) using NaOH or HCl.
Adjust final volume to 1 liter using double-distilled H_2O, autoclave, and then store.

9.3.2 Procedure for Making Dulbecco's Phosphate Buffer Saline with Ca^{+2} and Mg^{+2} (1 Liter)

Here is the composition of DPBS with Ca^{+2} and Mg^{+2}(Table 10).

Add all components to 800 ml distilled water and mix well.
Adjust final pH within (7.2–7.4) using NaOH or HCl.
Adjust final volume to 1 liter, using double-distilled H_2O, autoclave, and then store.

NB: Generally, Ca^{+2} and Mg^{+2} free PBS/DPBS is used to wash the adhesive cell culture containers before treatment with trypsin-EDTA because Ca^{+2} or Mg^{+2} may react with trypsin. However, since EDTA is already present with trypsin, it helps to chelate Ca^{+2} and Mg^{+2}.

9.4 Normal Saline

- **Normal saline** is the name for **0.89% sodium chloride (NaCl) solution** in double-distilled water.
- This solution is **called normal saline** because its osmolarity (308 mOsmol·L^{-1}(calc)) is nearly the same as that of blood. It contains 154 mEq·L^{-1} Na and 154 mEq·L^{-1} Cl.

To prepare normal saline the following requirements need to be met.

NaCl: 0.89 gm.
The final volume of double distilled water is 1000 ml.
The pH of normal saline is 4.5 to 7.0.

Table 10 Composition of Dulbecco's Phosphate Buffered Saline (**DPBS**) with calcium and magnesium

Name	Concentration (mM)	Amount (in g) for 1 liter
NaCl	136.9	8
KCl	2.67	0.2
KH_2PO_4	1.47	0.2
Na_2HPO_4 (anhydrous)	8.10	1.15
$CaCl_2 \cdot 2H_2O$	0.90	0.10
$MgCl_2 \cdot 6H_2O$	0.50	0.1

9.5 Alsever's Solution

- **Alsever's solution** was first used in 1941 by the American hematologist ***John Bellows Alsever***.
- Alsever's solution is a saline liquid used as an anticoagulant of blood (Table 11).
- An equal volume of Alsever's solution is gently and thoroughly mixed with the blood to prevent coagulation.

9.5.1 Role of Alsever's Solution

- The solution is frequently used as a blood cell preservative as it permits the storage of blood cells at refrigerator temperatures for nearly 10 weeks.
- The benefits of using Alsever's solution are its ability to support the antigenic life of red blood cells (RBCs), enabling its use in RBC screening, identification panels, as well as RBCs, drawn for serological investigation.
- Alsever's solution use is not restricted to human RBCs; it is also used to preserve RBCs from animals.
- Sheep and poultry are the most common animals that have their RBCs preserved in Alsever's solution.

9.5.2 Precautions and Limitations of Alsever's Solution

Like all solutions, Alsever's solution may have the following limitations:

- The solution should only be used for in vitro diagnostics purposes.
- The solution is light-sensitive, so should be stored in a dark place.
- Turbidity of the Alsever's solution generally indicates contamination which means the solution should not be used.
- Blood specimens showing signs of hemolysis or contamination should not be suspended in Alsever's solution as it may severely reduce the shelf life of the suspension.
- Bottles or vials of Alsever's solution which are leaking should be discarded.
- For the Alsever's solution to remain stable until the end of the shelf life, it should be stored in a fridge between (2–8) ^{o}C.

Table 11 Composition of Alsever's solution

Name	Amount (in g) per liter
D-glucose/ dextrose	2.05
Sodium citrate	0.8
Citric acid	0.055
NaCl	0.42

NB: During autoclaving, all solutions containing D-glucose can be charred at high temperatures. Therefore, D-glucose solution is prepared in minimum water and filter sterilized with a Millipore membrane filter. The remaining ingredients are added to water having a final volume of 1 liter minus the D-glucose solution volume. The filter-sterilized D-glucose solution is later added to the autoclaved solutions to get the final volume of reconstituted 1 liter BSS. HEPES and $NaHCO_3$ are common buffers used to balance the pH of the culture medium.

10 pH Maintenance of the Cell Culture Medium

pH is the negative logarithm of H^+ concentration. pH ranges from 0 to 14. At 25 °C, pH 7 is neutral. At 25 °C, solutions with a pH less than 7 are acidic and solutions with a pH > 7 are basic. pH maintenance of a cell culture medium is highly essential.

- All cell culture media are checked for pH before adding the supplements.
- The pH of all culture media is checked with pH paper, but not with a pH meter as a medium can interfere with an electrode of a pH meter.
- The pH of the medium is adjusted with NaOH and HCl.
- All mammalian cell culture medium pH is maintained in a lower alkaline range like blood that is, **between 7.2 and 7.4 (while human cell cytoplasm pH is 7.2, blood pH is 7.4)**.
- This is called the **physiological pH range** as this is the optimum pH for mammalian cells and helps the cells to maintain proper ionic balance and keep the optimum activity of cellular enzymes, hormones, and growth factors in cell cultures.
- **In a CO_2 incubator, the regulation of mammalian cell culture medium pH uses the buffering system as a bicarbonate – 5% CO_2 system**.
- To detect the pH of the culture medium visually, **phenol red, a chemical pH indicator** is added to most commercially available culture media. Phenol red indicates medium pH changes by cell metabolites by readily changing its color.
- **In presence of phenol red, the alkaline pH of the medium is detected by light pink or orangish color whereas acidic pH is determined by its yellow color.**
- An acidic pH below 6.8 (yellowish color) indicates either bacterial contamination or retarded cell growth in the spent medium. So, when the pH of the medium falls below 7.0, the medium needs to be changed as early as possible (Howorth 1975).

NB: Phenol red has structural similarity with estrogens and estrogen-like phenolic/polyphenolic compounds. Therefore, phenol red must be removed while experimentally working with estrogens/other steroids/phenolic compounds.

10.1 Maintenance of Osmolarity/Osmolality of the Cell Culture Medium

- Mammalian cells need an isotonic environment. So, the cell culture medium must maintain isotonic conditions.
- The ***ideal osmotic pressure of the cell culture medium intended for culturing the human cells is 290 mOsm·kg^{-1}***.
- This osmotic pressure is needed as it is the **osmotic pressure of human plasma**.
- However, in most mammalian cells, osmotic pressure ranges between (260–320) mOsm•kg^{-1}.
- The osmotic pressure of a mouse plasma is about **320 mOsm•kg^{-1}**.
- Mainly various salts, glucose, and amino acids maintain the osmotic pressure of mammalian cell culture medium to an isotonic level (**~300 mOsm**).
- The **osmolality** of the **culture medium** should be similar to the **osmolality** of the physiological environment.
- While osmolality can affect both cell growth and function, the medium hyper-molality can shrink the cells and hypo-molality can make them swell.
- Thus, the culture medium is always kept isotonic and all other buffers and solutions are maintained at this level only (Waymouth 1970).

11 Antibiotics and Antimycotics

- By definition, **antibiotics are substances produced by a group of microorganism (e.g., *Penicillium notatum*, a yeast that produces antibiotic penicillin) and acts against another group of microorganism (e.g., Gram-positive bacteria) at low concentration**.
- Similarly, antimycotics are substances produced by the microorganisms such as yeast and prevent the growth of fungus or molds.
- Antibiotics and antimycotics are slowly degraded at 37 °C or room temperature and perhaps in refrigerators too.
- So, since there is a life span of antibiotics and antimycotics, they are required to be added fresh in every mammalian cell culture medium to prevent the growth of the contaminating microorganisms (bacteria and fungus, respectively).
- **Penicillin, streptomycin**, and **gentamicin** are common **broad-spectrum antibiotics** working against bacteria.
- **Nystatin** and **amphotericin B** are used as **antimycotic agents** against yeast and fungi.
- Three classes of antibiotics kill **mycoplasma** when used at relatively low concentrations: **tetracycline, macrolides, and quinolones**.
- Table 12 describes some antibiotics and antimycotics, their storage temperature, the final concentration needed for the cell culture medium, and stability at 37 °C.

Table 12 Description of antibiotics and antimycotics, their final concentrations, storage temperature, and stability in a CO_2 incubator

Name	Storage temperature	Concentration (final)	Stability in CO_2 incubator (37 °C)	Antibiotic/ antimycotic
Penicillin G Benzylpenicillin	−20 °C	100 U·ml^{-1}	3 days	Antibiotic
Ampicillin sodium salt	4 °C	100 μg·ml^{-1}	3 days	Antibiotic
Ciprofloxacin hydrochloride	−20 °C	10 μg·ml^{-1}	Not known	Antibiotic
Tetracycline hydrochloride	−20 °C	10 μg	4 days	Antibiotic
Gentamycin sulfate	4 °C	50 μg·ml^{-1}	5 days	Antibiotic
Neomycin sulfate	-20 °C	50 μg·ml^{-1}	5 days	Antibiotic
Polymyxin B	4 °C	100 U·ml^{-1}	5 days	Antibiotic
Hygromycin B	4 °C	500 μg·ml^{-1}	Not known	Antibiotic
Kanamycin sulfate	−20 °C	100 μg·ml^{-1}	5 days	Antibiotic
Streptomycin sulfate	−20 °C	100 μg·ml^{-1}	3 days	Antibiotic
Chloramphenicol	−20 °C	5 μg·ml^{-1}	5 days	Antibiotic
Puromycin dihydrochloride	−20 °C	20 μg·ml^{-1}	Not known	Antibiotic
Nystatin	−20 °C	100 μg·ml^{-1}	3 days	Antimycotic
Amphotericin B	−20 °C	2.5100 μg·ml^{-1}	3 days	Antimycotic

The following are the cautions regarding the use of antibiotics/antimycotics in a mammalian cell culture medium:

- Antibiotics/antimycotics target specific molecules in the microorganisms. For instance, penicillin attacks penicillin-binding proteins (**PBPs**), a transpeptidase enzyme prevailing in the peptidoglycan layer of the bacteria. PBPs help in the cross-linking of the peptidoglycan layer.
- The PBSs are not produced by mammalian cells. However, a very high penicillin concentration may nonspecifically affect the cultured mammalian cells and express toxic effects. Therefore, always use a low but sufficient concentration of antibiotics/antimycotics that will affect only the microorganisms and not the cultured mammalian cells.
- The addition of serum neutralized many of the exerted toxic effects on the cultured mammalian cells.
- *As discussed in the earlier paragraphs,* ***it is best either not to use antibiotics in a serum-free culture medium or to use them at a 5–10 times lower extent as compared to serum-containing medium.***
- Drug (antibiotic/ antimycotic)-resistant microorganisms could pose significant difficulty in mammalian cell culture.

- Since antibiotics/antimycotics are temperature-sensitive, so care must be taken during repeated freeze-thawing of antibiotics or proper replacement of cell culture medium after stipulated days of incubation at 37 °C in a CO_2 incubator.

NB: Stock antibiotics and antimycotics should be stored at − 20 °C in several aliquots.

- Antibiotics/antimycotics are not useful against virus contamination in cell culture. While contaminations with various viruses are rare, those by mycoplasma are a serious obstacle in mammalian cell culture since cell culture serum is often infected with mycoplasma. While various interferons will regulate virus growth, ***mycoplasmin*** can regulate mycoplasma growth. Peptidoglycan synthesis inhibitors such as penicillin cannot be useful against mycoplasmas because mycoplasmas do not have any cell wall (Perlman 1979).

12 Conclusions

Mammalian cells need various nutrients and other materials for culture, growth, and maintenance. The materials commonly needed for the mammalian cell culture are cell-dissociating agents, cell adhesive agents, various cell culture media, and various types of buffers. The cell-dissociating agents are necessary for dissociation and the isolation of mammalian cells from the tissues and organs. Once the cells are dissociated, the next step is to seed the isolated mammalian cells into the cell culture vessels containing the cell culture medium. Since most mammalian cells are adhesive in nature, cell adhesive agents may be added to the mammalian cell culture containers which are made up of polystyrene. After the treatment of the polystyrene-containing culture vessels with the adhesive agents, cells suspended in a cell culture medium are added to the culture vessels. The cell culture medium contains all the necessary materials necessary for the culture and growth of the cells. However, since different cells may need different amounts or types of various nutrients, different types of cell culture media are formulated accordingly. Finally, various buffers are necessary for the isolation, culture, and maintenance of mammalian cells. This chapter described all these materials necessary for the isolation, culture, and maintenance of mammalian cells.

13 Cross-References

► Culture of Continuous Cell Lines
► Culture of Neuron and Glia Cells
► Isolation and Primary Culture of Various Mammalian Cells
► Primary Culture of Immunological Cells
► Stem Cell Culture and Its Applications

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Microbial Contamination of Mammalian Cell Culture

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_5-1

Abstract

Contamination is commonly used as a term for the presence of undesired microorganisms, unwanted mammalian cells, and various biochemical or chemicals present in the cell culture medium and thus affects the physiology and growth of the desired mammalian cells. Since microbes are present everywhere in the environment including the specific regions of the human body and their doubling times are much faster than the fastest growing mammalian cells, microbial contamination poses a major challenge to the mammalian cell culture. The most important microbes that contaminate the mammalian cell culture are ***bacteria, fungi (yeast/molds), mycoplasmas, viruses, and protozoans.*** While bacterial and fungal contaminations are the most common and easily visible, mycoplasma and virus contaminations are hard to detect and cannot be confirmed by naked eyes. Contamination by protozoans is very rare. Besides microorganisms, another subject that may contaminate mammalian cell culture is ***unwanted mammalian cells*** (e.g., HeLa cells). The reason for unwanted mammalian cell contamination is mislabeling during culture, handling of more than one cell at a time, removal of the label from the culture vial in liquid nitrogen, and so on. Contamination poses a major threat in cell culture technology since in most cases contaminated cells need to be discarded which causes a huge loss of money and waste of manpower. It is therefore highly essential not only to create a germ-free environment in the cell culture laboratory but to remain highly cautious during the culture procedure. It is therefore of utmost importance to gain knowledge regarding various types of contaminations, their identification, and possible elimination.

Keywords

Mammalian Cell Culture Contamination, Bacteria, Fungi (Yeast/ Molds), Mycoplasmas, Viruses, and Protozoans · Cross-Contamination by Other Mammalian cells

1 Introduction

The in vitro culture of mammalian cells is a crucial requirement of biomedical research. Prompt readiness of aseptic techniques is highly critical for ensuring that only the required cells keep growing in the culture. Despite best efforts, contamination still prevails pretty commonly in all cell culture laboratories. Mammalian cell culture contamination is the presence of unwanted materials such as microorganisms, mammalian cells other than the desired cells that are being cultured, and various biochemical or chemicals unnecessarily present in the cell culture medium and thus affect the physiology and growth of the desired cells. In any research laboratory, contamination is not merely an occasional irritation, but it can lead to a loss of significant resources including time and money. Ultimately, contamination can affect the working ability of a research group or a particular scientist. Several publications sometimes are shatteringly withdrawn owing to the concerns about retrospective sample contamination or reported results being artifacts. In biopharmaceutical manufacturing, contamination can be even more risky or deleterious, ultimately leading to the discard of entire production runs and causing irreparable loss of money and manpower hours.

Contamination is a major issue every mammalian cell culture laboratory faces today, starting from the research laboratory in the academic and industrial domains. Ultimately, once contamination(s) is established it has the potential to spread through the air in the laboratory, thereby making all cultured cells vulnerable within the laboratory and inevitably, enhancing the infection probability among the laboratory personnel via aerosol generation. While pipetting is the most important reason for aerosol generation, several other human activities could also generate aerosol(s). Some laboratory activities generating aerosols include vortexing, pipetting, syringe-mediated fluid transfer or injection opening sample containers, and centrifuges. The aerosol production in the Clinical Biochemistry Laboratories (**CBLs**) is recognized by the Center for Disease Control (**CDC**), Atlanta, USA.

It is, therefore, extremely important, to understand the sources which could contaminate the cell culture medium. The major contaminating microbes for mammalian cell culture medium are bacteria, fungi (yeast/mold), mycoplasmas, and protozoa. Additionally, animal viruses, which could be living or nonliving (in between life and death), could also contaminate a cell culture medium. Contamination caused by other unidentified mammalian cells is another source of external perturbation. Since microorganisms prevail everywhere in the environment including aerosol or air, land, or even the surfaces of various cell culture instruments and water including cell culture medium or even medium constituents such as serum, they can easily contaminate the cell culture. Microorganisms grow very fast,

compared to the doubling time of various mammalian cells, (even the fastest-growing mammalian cells may take 10–12 h to double). Contrary to this, various bacteria double their population in just 20–30 min. Thus, if and when contamination of a mammalian cell culture medium by bacteria, rapidly proliferates, it leads to a vanishing of all the nutrient constituents along with overwhelming the mammalian cells. In addition, the contaminated microorganism may change the pH of the culture medium and have a seriously debilitating effect on the growth, metabolism, morphology, and overall physiology of the mammalian cells.

It is desirable that if or as and when microorganisms contaminate the mammalian cell culture, they must be detected timely and easily so that suitable steps could be taken for their elimination. However, not all microbial contaminations are easy to perceive via the naked eye. For example, in general, contamination, followed by bacterial growth in a mammalian cell culture medium mostly changes the culture medium pH. Since phenol red added to the medium acts as a pH indicator, it can be observed through the naked eye. However, as and when the bacteria further grow, the turbidity of the culture medium changes. This turbidity change along with color changes of the medium indicates probable contamination with the microorganism(s). Similarly, contamination and growth by the fungi in the mammalian cell culture medium can be easily detected by the naked eyes. Unlike bacterial contamination-driven pH changes in the cell culture medium, mold contamination usually does not change the pH, at least until the advanced growth stages. Therefore, mold contamination at the initial stage does not change the pH of the mammalian cell culture medium. However, detection of viral and mycoplasma contaminations cannot be accurately made through the naked eye because of their small size, also because of no changes in the medium pH. However, some observations claimed mycoplasma detection as small black-black dots through inverted microscopes.

The contaminating microorganism(s) and/or infecting agents (e.g., viruses) vary in their shape, size, and growth pattern in a mammalian cell culture medium besides responding to antimicrobial agents. For example, viruses are so small (e.g., as low as 20 nm in AAV DNA virus) that they can pass easily through the **membrane filters** used in the sterilized mammalian cell culture medium. Contrary to this, mycoplasmas are larger (100 nm) in size than viruses but are smaller than bacteria (200–300 nm). Thus, mycoplasmas can pass through membrane filters easily (pore size 0.22–0.45 μm). Additionally, while **antibiotics** (e.g., penicillin, ampicillin, streptomycin, etc.) are used to control contaminating bacterial growth in a mammalian cell culture medium, **antimycotics** (e.g., amphotericin B, nystatin, etc.), **plasmins** are used as antimycoplasmic agents and **interferons** are used as an antiviral agent. ***Thus, it is highly essential to identify the contaminating microorganism(s)***. In this connection, profiling of ***Short tandem repeats (STRs)*** is one of the most important and widely used methods for the identification of specific microorganism (s). The protocols of STR profiling in the human cell lines are available on the website of The American National Standard Institute (**ANSI**). Other methods used for the characterization and identification of contaminated cells in the mammalian cell culture are isoenzyme analysis, karyotyping, and DNA barcoding. Another method that is utilized for profiling contaminated cells is polymerase chain reaction (**PCR**)

and restriction fragment length polymorphism (**RFLP**). Though PCR-RFLP is less expensive it is time-consuming.

Thus, understanding the mechanism for limiting and preventing contamination is of paramount importance. Indeed, complete elimination of cell culture contamination is rather very difficult, but cautious behavior of the laboratory personnel can significantly decrease or in some cases, entirely nullify the chance happening of contamination. Some important procedures to prevent or reduce the contamination chances of mammalian cell culture include wearing gloves, regular cleaning of lab coats, and working only inside the laminar flow hood along with its scientifically aseptic use. Working on one cell line at a time minimizes the exposure of other cells to nonsterile environments (e.g., during microscopic visualization), use of antibiotics and antimycotics should be made only if it is necessary. For the routine purpose, spray everything with 70% ethanol, and use sterile reagents, labware, and filter tips that are changed often apart from frequent inspection of cells. One should observe good lab practices while working, i.e., cleaning the incubator, water bath, laminar flow, the laboratory premises regularly, monitoring the quality control of cultured cells, and efficient biohazard disposal. Finally, the microscope should be used to examine all tissue culture vessels for any sort of contamination (tiny dots of bacteria or stings of hyphae from fungi/mold, etc.). Remove all infected vessels to an appropriate laboratory where no tissue culture is done or rather destroy them (Fogh et al. 1971; Fogh 1973; Stacey 2011).

2 Sources of Microbial Contamination in Mammalian Cell Culture

Microorganisms are present everywhere including the solid surfaces (e.g., instruments such as CO_2 incubators, centrifuges, laminar flow working tables, etc.), contaminating the air inside the cell culture laboratory besides adulterating the cell culture medium (via serum, trypsin, etc.). Microorganisms love to grow in the mammalian cell culture media since they are very rich in all kinds of nutrients. Thus, despite several steps being taken to grow mammalian cells without any contamination, there may still be chances of contamination.

The major sources(s) of contamination are as follows:

- The source cells may be unknowingly contaminated by the vendors before supplying to the laboratory.
- Mammalian cells, tissues, and blood materials such as serum are the sources of various pathogenic **virus contamination**.
- Source serum may be contaminated with mycoplasma. In general, it is observed that fetal bovine serum (**FBS**) or fetal calf serum (**FCS**) is a rich source of **mycoplasma contamination.**
- **Bacterial contaminations** manifest significantly via aerosol generation on the surfaces of various instruments, or even water.

- The airborne microbes can easily enter the mammalian cell culture containers, contaminate them and outgrow desired cells in culture if the good cultural practices including "***maintaining sterility from start to end***" (in a cell culture process) are not followed.
- Endotoxins are in fact, lipopolysaccharides containing by-products of Gram-negative bacteria, which are perhaps the major chemical contaminants in cell culture systems. Common prevailing habitats of endotoxins include water, sea, and some culture additives.
- Depending on the presence extent, too high endotoxin content can be significantly deleterious for the growth and performance of cultures, being the cumulative source of experimental variability. However, ever since the use of cell culture produced therapeutics, such as hybridomas and vaccines, serious caution must be exercised to arrest the endotoxin content of culture systems to minimal proportions.
- Residues from germicides are used to disinfect incubators, laboratory equipment, glassware, and labware and remove gaseous impurities in CO_2 incubators, all of which are deleterious to cell culture.
- Regarding various kinds of **fungal contaminations, the one with** *Aspergillus* is predominant. Some studies report that the source of *Aspergillus* contamination in a mammalian cell culture medium is the infected oral cavity of the laboratory personnel.
- Laboratory working surfaces and the air are already contaminated with fungal spores generated while the working duration of the previous person. Of note, generally, a mammalian cell culture laboratory should not be used to culture any microorganism, and therefore, it must be dedicated solely to mammalian cell culture.
- The filters used for medium sterilization might not work properly due to defects or date expired filters.
- The cell culture containers (e.g., Petri plate) were not sterilized due to various reasons including missed sterilization.
- Rough handling of cells/cell culture materials leads to accidental contamination.
- In general, contamination of the mammalian cell culture with protozoa such as *Giardia intestinalis*, and *Entamoeba histolyticum* is very rare.
- While working with more than one cell line at a time, there are significant chances of cross-contamination or mislabeling. It is also observed that liquid nitrogen deep-freezing may remove the labeling of stored cell vials, which may lead to misidentification (Barile and Kern 1971; Fogh et al. 1971; Fogh 1973; Stacey 2011).

3 Identification of Cell Culture Contamination

- Of the various types of microbial and other infectious agents, contaminating the mammalian cell cultures, in some cases, the contamination can be observed easily through the naked eyes while some of these may not be observed with the naked

eyes. Bacteria and fungi (molds/yeasts) are included in the first group, while viruses and mycoplasmas are included in the second group.

- Another important source of bacterial and fungal contaminations is their abundance in the environment including land, water, and air, manifolding the high-level contamination chances of the mammalian cell culture medium.
- Since bacteria and fungi cause changes in the turbidity as well as in the pH of the medium that can be easily visible through naked eyes.
- On the other hand, contamination with mycoplasmas and viruses cannot be detected through the naked eyes since it rarely results in pH or turbidity changes in the medium. However, under an inverted microscope, mycoplasma is viewed as a small black dot in the cytoplasm of the mammalian cells and can be identified and separated from cell debris by their specific size, causing no translocation amidst shaking of cell culture medium.
- It is not possible to detect the contamination with various cells, other than the cultured cells of interest through naked eyes. However, under a microscope, one can predict the contamination of other cells based on the cell's morphological features. However, for confirmation of contaminated cells, several biochemical, molecular biology, and genetic tests are necessary.

Some of these strategies include the following:

- **Visual inspection** with the naked eye. For example, the presence of a cloudy film may indicate bacterial contamination.
- **Microscopic examination** (including electron microscopy), can help reveal many different undesired microbial contaminants, such as bacteria, viruses, and yeast.
- **Testing the pH**: Usually, heavy contamination by yeast may increase the culture medium pH contrary to that of bacterial contamination which decreases the same, thereby enabling distinctive recognition.
- **More advanced testing** employs ELISA or enzyme-linked immunosorbent assay, in which proteins called antibodies are attached to antigens (the potential contaminants) to reveal the latter's presence.
- There are several specific identification tests for each of the contaminants microorganisms which are discussed in the specific section alongside the contamination aspects of each microbe (Ryan 2008).

3.1 Distinction Between Cell Debris and Contamination

- Cell debris usually can be eliminated by **washing the cells with a buffer** while contamination cannot.
- The medium color with debris does not change (pink/salmon) while contamination results in acidification and the color turns yellow.
- **A hazy or cloudy medium** always means contamination by microorganisms.

4 Types of Microbial Contamination

As discussed so far, several microbes can contaminate mammalian cell culture, most easily identifiable among which are bacteria and fungus (mold/yeast). The presence of mycoplasmas, viruses, and cross-contamination by other mammalian cells, is rather hard to detect. In general protozoan, contamination is rare (Lincoln and Gabridge 1998).

Ahead is a brief discussion of agents that causes mammalian cell culture contamination:

1. Bacteria Contamination
2. Fungi (Molds/Yeasts) Contamination
3. Mycoplasmas Contamination
4. Viruses Contamination
5. Protozoans Contamination
6. Cross-Contamination by Nonnative Cells

Here is a brief discussion of the contamination by the above microbes:

4.1 Bacterial Contamination

- Bacterium (singular) or bacteria (plural) are small (average ~0.2–2 μm in diameter), microscopic, single-cell prokaryotes that can't be grouped as plant or animal cells.
- Depending upon the type, the single-cell bacterium may form pairs, chains, or clusters.
- Bacteria are known to exist in various shapes, leading to recognized as **Coccus** (spherical, e.g., *Staphylococcus aureus*), **Bacillus** (rod, e.g., *Bacillus subtilis*), and **Spirillum** (*coil*-like, e.g., *Treponema pallidum*) structure.
- Based on the Gram stain (discovered by ***Christian Gram***), bacteria can be either **Gram-positive** (e.g., *Staphylococcus aureus*, takes the crystal violet stain) or **Gram-negative** (e.g., *Escherichia coli,* stains by pink color safranin).
- Bacteria are found in almost every terrestrial habitat, i.e., soil, rock, oceans, and even arctic snow. Some live in or on other organisms including plants, animals, and humans. Trillions of bacteria are present in the human body, exclusively in the digestive tract.
- Some bacteria are autotrophs, some maintain symbiotic relations with the host while still others live just like parasites.
- On average, a bacterium may double, its initial population in just 20–30 min.
- Because of their ubiquity, size, and fast growth rates, bacteria, along with yeasts and molds, are the most commonly encountered cell culture contaminants.
- ***Escherichia coli*** *and* ***Staphylococcus aureus****,* ***bacillus,*** and ***enterococcus*** **species are some of the most commonly contaminating bacterial species** (MacPherson and Allner 1960).

4.1.1 Detecting Bacterial Contamination

- Bacterial contamination is usually manifested by a sudden change in pH, apart from a pink to yellowish change of medium phenol red color.
- Of note, phenol red which is added to the mammalian cell culture medium acts as a pH indicator (Fig. 1 depicts the color changes of the cell culture medium of a T-75 flask after contamination with bacteria).
- Apart from the above, other characteristic hallmarks of bacterial contamination include the development of cloudiness (turbidity) in the medium, sometimes with a slight whitish film on the cell surface of plates, dishes, or at the bottom of medium bottles that typically dissipates when the vessel is moved. For a better understanding, readers are suggested to refer the section: Antiseptic Techniques, Biological Contamination in the Handbook of Cell Culture Basics, GIBCO Invitrogen.
- There is a distinct and clear observation of contaminated bacterial morphology while observing the contaminated area from low power (100×) to high power/higher magnification (400×).
- When viewed through a low power, the contaminating bacteria may look like small black dots in the cytoplasm whereas, in higher magnification, distinct morphological observation (e.g., round-shaped coccus versus rod-shaped bacillus) or even motility of the live bacteria may be noticed.
- Bacteria can usually be distinguished from medium components such as serum proteins and others by a characteristic and regular particulate morphology contrary to that of an irregular one for serum protein cryoprecipitate (McHugh and Tucker 2007).

4.1.2 Controlling Bacterial Contamination of the Mammalian Cell Culture

Generally, if we have adequate stock vials in liquid nitrogen, one can comfortably discard the bacterial contaminated cultures. However, if one does not have any alternative cells, it is better to treat the cell culture with the following antibiotics (Table 1).

Fig. 1 T-75 flask, depicting bacterial contamination (**a**) contaminated texture, and (**b**) non-contaminated texture. (Figure made after seeking inputs from Bratos-Perez et al. (2008))

Table 1 Antibiotic solutions for bacterial growth inhibition in a culture medium

Antibiotic	Concentration	Effective against
Penicillin	50–100 IU	Bacteria
Streptomycin sulfate	50–100 μg	Bacteria
Neomycin sulfate	50 μg	Bacteria
Polymyxin B sulfate	100 U	Bacteria
Gentamicin sulfate	5–50 μg	Bacteria, including mycoplasma
Kanamycin sulfate	100 μg	Bacteria, including mycoplasma

NB: Penicillin/streptomycin mixture is the most common antibiotic mixture used to arrest bacterial contamination of a cell culture medium. Some investigators may prefer gentamycin instead of penicillin/streptomycin. Similarly, many times, a plasmon is employed owing to its effectiveness against bacteria and mycoplasma.

4.2 Fungal Contamination

- A **fungus** (Plural: Fungi) is a eukaryotic organism with a distinct separate kingdom from the kingdom of protists, plants, and animals. Fungi are widely distributed on earth, having significantly diverse environmental and medical effects. While some fungi are free-living in soil or water, others form parasitic or symbiotic associations with plants or animals.
- The kingdom is broadly divided into three groups, i.e., molds, yeasts, and mushrooms.
- Structurally, the most important characteristic of the fungal cell wall is **chitin**.
- The fungal cell membrane contains **ergosterol,** equivalent to the cholesterol in the mammalian cell's plasma membrane.
- Of note, various antifungal drugs that are used against pathogenic fungus **interfere with ergosterol** and plasma membrane synthesis.
- Four fungal species ***Aspergillus, Mucormorale***, ***Cryptococcus, and Candida*** infect the human body, resulting in diseased conditions namely **aspergillosis, mucormycosis, cryptococcosis, and candidiasis,** respectively.
- Cell culture fungal contamination is dominated by *Aspergillus* species. ***M. morale*** *is* reported as the most common contamination source that can spread from the human oral cavity. Thus, the lab workers are exclusively affected by ***M. morale*** *is* contamination.
- Several methods are used to detect or diagnose fungal pathogens.
- In case, fungal contamination is not visibly detected, the direct microscopic imaging of the culture may give us the first clue regarding the fungal contamination of the culture. For biochemical tests, 1,3-β-D-glucan or galactomannan from the fungal cell is detected. Of note, galactomannan is a polysaccharide antigen found primarily in the cell wall of *Aspergillus* species and sometimes this fungus predominantly affects the cell culture. Various immunological and molecular biology tests can be used to confirm not only the specific fungal infection but

also for determining the specifically contaminated fungal strain. While the immunological tests include ELISA and antigen-antibody dependent serological tests), the molecular biology tests include polymerase chain reaction (**PCR**) and real-time PCR (**RT-PCR**) including multiplexing, restriction fragment length polymorphism (**RF-LP**), matrix-assisted laser desorption ionization-time of flight mass spectrometry (**MALDI-TOF-MS**) and **Lab-on-chip or INN.** Finally, DNA barcoding with dual loci such as ITS and *TEF1*α offers optimal accuracy for species identification of the isolates (Cour et al. 1979; Ascioglu et al. 2002).

The three major groups of fungi are as follows:

MOLDS: These are multicellular and filamentous fungi.

MUSHROOMS: Macroscopic, large fruiting bodies prevailing in a filamentous texture. Generally do not contaminate the mammalian cell culture and therefore are not of much interest to the mammalian cell culture.

YEASTS: These are unicellular microscopic fungi, having characteristics of eukaryotic cells, but possessing bacteria-like plasmids. The plasmids present in the yeast are called **2 μm episomes**. **For recombinant DNA technology/DNA or gene cloning/genetic engineering, works yeast may be transfected with various artificial vectors called yeast artificial chromosomes (YAC).**

NB: Mammalian cell culture is exclusively contaminated by molds and yeasts.

4.2.1 Characterization of Mold Contamination

- Molds are recognized as multicellular eukaryotic microorganisms having filamentous structures.
- Some of the pathogenic molds are ***Aspergillus and Mucorales***.
- Color-wise molds can be black, white, orange, green, or purple, having an equal probability of living indoors as well as outside.
- Molds look like fizz or threads and thrive on moisture, reproducing via lightweight spores that travel through the air (Fig. 2 depicts the inverted microscopic view of mold contamination in mammalian cell culture).
- Mold invasion is harmless if limited to smaller extents.
- However, after landing on a damp spot in and around our homes, the molds begin to grow. They release spores that anybody, including lab personnel, might inhale. If somebody is sensitive (allergic) to mold and inhales a lot of spores, he/she could fall sick.
- The filaments of a mold are popularly known as **hyphae** and a collection of hyphae that are genetically identical in nuclei is called **mycelium.**
- Like bacteria, molds also form spores that can survive in harsh and inhospitable environments, remaining dormant for a long duration.
- Spores from the molds can contaminate the cell culture medium.
- Under favorable or optimum conditions, the spores may form new molds.
- A mold-contaminated media forms a cloudy/dark solution.
- The garbage-like smell may emerge from the mold-contaminated culture containers.

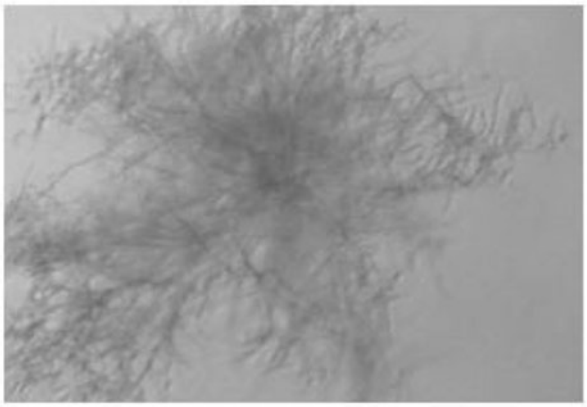

Fig. 2 Inverted microscopic view of mold growth in a mammalian cell culture container. (Included with permission: https://unclineberger.org/tissueculture/contaminant/funguscontam/)

- The growth of molds in the mammalian cell culture medium begins slowly, gradually they form while, or black, or another colored mycelium which floats in the culture medium and can be observed distinctly through the naked eye.
- While at the initial stages of mold contamination, no changes in the medium pH occur. However, at the advanced growth stage, the turbidity of the medium may result in a pH increment.

4.3 Contamination with Yeast

- Yeasts belong to the fungal kingdom of unicellular **eukaryotic microorganisms.**
- At present, there are 50 genera and 1000 yeast species that have been identified. *Saccharomyces cerevisiae, commonly known as Baker yeast* is the most well-studied yeast, used in the bread industry.
- The yeast termed **red rice** is a mold ***Monascus purpureus***.
- The shapes of the yeasts are spherical while the size varies from a few micrometers (typically) to 40 μm (rarely).
- These microorganisms reproduce both asexual **(budding)** as well as sexual (**conjugation**) modes.
- Yeast may also reproduce via fission or pseudohyphae.
- Depending on the specific species and strain, yeast may be useful in various industries or even harmful by promoting certain diseased conditions.
- In general, yeasts are used for sugar fermentation and wine production (ethyl alcohol/C_2H_5OH).
- Since yeast are simple (single-cell eukaryotes) and exhibit some similarities with mammalian cells, they are used in genetics and cell biology experiments. For example, yeast artificial chromosomes (**YAC**) which are produced from **2 μm plasmids** or **episomes,** are widely used for the cloning and expression of eukaryotic genes.
- ***S. boulardii,*** a type of yeast is used as a constituent in probiotic supplements.

- Another yeast, ***Clostridium difficile*** suppresses bowel movement in diarrhea-predominant IBS patients. This diminishes the incidence of antibiotics, travelers, and HIV/AIDS-associated diarrheas.
- ***Candida*** species of yeasts are recognized as ***opportunistic pathogens***. **Candidiasis** is an infection with various *candida* strains in the mucus membrane of the oral cavity and vagina.
- Besides **candida, *Cryptococcus*** is another kind of yeast surrounded by a thick coating of polysaccharides called a capsule. *Cryptococcus* not only can contaminate a mammalian cell culture but also causes a pathogenic condition in humans, which is **pulmonary cryptococcosis**. However, *Cryptococcus* infection to the mammalian cell culture may be at a lower extent compared to other fungal infections (Fig. 3).

4.3.1 Detecting Yeast Contamination

- Similar to bacterial contamination, cultures contaminated with yeasts become turbid (cloudy), particularly in the advanced stages.
- At this stage, yeasts generally cause the growth media to become very cloudy or turbid.
- Yeast contaminated culture smells like "***baking bread***."
- Low to moderate level contamination with yeast does not change the pH of the culture medium. However, high-level contamination indeed changes the medium pH to alkaline.
- The 100×–400× magnification of the phase-contrast microscope is used to observe the yeasts.
- Visualization of mild yeast contamination through a phase-contrast microscope is witnessed as ovoid bright particles between the cells.
- They can be seen as single cells or as chains or branches, called as "***String of Pearls***."
- The mammalian cells will be dead and poorly spread in presence of yeast contamination.

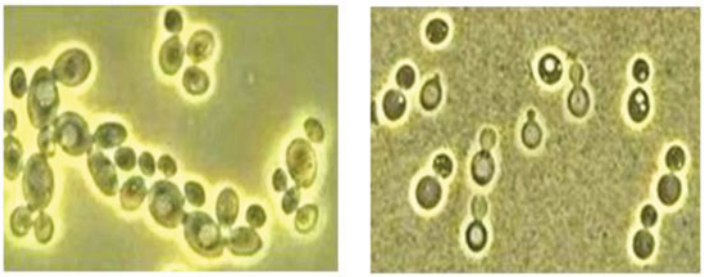

Fig. 3 Microscopic view of the yeasts morphology in culture media, (**a**) *Saccharomyces cerevisiae* and (**b**) *Cryptococcus albidus.* (Figure incorporated from: http://archive.bio.ed.ac.uk/jdeacon/microbes/yeast.htm)

- Table 2 represents the basic characteristic differences between yeast and mold contamination in mammalian cell culture.

4.3.2 Elimination of Yeast Contamination

Generally, yeast-contaminated culture must be discarded. However, if the culture is very precious, the culture may be treated with either of the following antimycotics (Table 3 describes the name and concentration of antifungal agents, including antiyeast agents).

Table 2 Comparisons between mold and yeast culture contamination

Basis for comparison	Yeast	Mold
Meaning	Yeast is a type of single-cell microorganism, that falls under the fungal kingdom	Molds are multicellular microorganisms, usually colorful, and also fall under a fungal kingdom grown as multicellular filaments called hyphae
Cellular organization	Unicellular	Multicellular
Shape	Filamentous or threadlike	Round or oval
Presence of hyphae	Pseudohyphae, i.e., they do not have true hyphae	Hyphae (microscopic filament)
Presence of spores	Nonsporous fungi	Sporous fungi
Types	There are 1500 types of yeast	There are 400,000 types of molds
Mode of reproduction	Asexual through mitosis, the most common methods include budding and spore formations	Sexual or asexual
Color	Colorless and smooth (usually white)	Colorful, wooly, and fuzzy (green, orange, black, brown, pink, purple)
Aerobic/ anaerobic	Yeast can grow in aerobic as well as anaerobic conditions	Molds grow only in aerobic conditions
Uses	Yeast is useful in beverage production like alcohol and also in baking bioremediation, food additives, etc.	Useful in biodegradation, food production (cheese), and in making antibiotics
Health hazards	Yeast may weaken the immune system of the body, alongside contributing to asthma and Crohn's disease	Molds may cause allergies and respiratory problems
Examples	*Saccharomyces cerevisiae* (baking yeast), *Cryptococcus neoformans,* etc.	*Mucor, Penicillium, Rhizopus*, *Aspergillus*, etc.

Table 3 Workable concentrations of *Antimycotics* to control fungal and yeast contamination

Antimycotic solutions	Concentration (per mL)	Effective against
Amphotericin B	2.5 μg	Fungi including yeast
Fungizone	15 μg	Fungi including yeast
Nystatin	100 U	Fungi including yeast

4.4 Mycoplasma Contamination

- In 1956, ***Robinson and coworkers*** first detected mycoplasmas/pleuro-pneumonia-like organisms (**PPLO**) in the cell cultures. They were attempting to study the effects of PPLO on HeLa cells when they discovered that the control HeLa cultures were already contaminated by PPLO (Robinson et al. 1956).
- Later on, they also observed that the other cell lines being concurrently used while working with HeLa cells were also contaminated with mycoplasma(s).
- Mycoplasmas are included in the kingdom **Monera,** and the class ***Mollicutes***. **Mollicutes** are **Gram-positive bacteria** with a **0.58–2.20 Mb** genome. Presently more than **100** mycoplasmas **species** are known.
- These microorganisms are characterized by the missing **cell walls** and their **plasma-like texture.**
- At least 11–15% of cell cultures being tested by **FDA, ATCC,** and various companies in the United States, are currently infected by mycoplasmas.
- Mycoplasma contamination levels in some cultures in Europe are extremely high, i.e., between 25% and 40%.
- In some cultures, mycoplasma contamination level in **Japan** has been as high as 80%.
- The discrepancy between various countries in the world including the United States is likely because of different testing programs being used.
- Analyses inferred that laboratories that routinely test for mycoplasma contamination have a much lower risk of facing contamination. However, once detected, contamination can be contained and eliminated.
- Thus, it is recommended that testing for mycoplasma should be performed at least once on monthly basis. There is a wide range of commercially available kits for screening this contamination.
- At present, mycoplasmas are considered as ***smallest self-replicating organisms.***
- Mycoplasmas have a small size with 0.2–0.8 μm diameter. They have a flexible cell membrane. This small size and cell membrane flexibility allow mycoplasma to pass through commonly used antibacteriological filters with nearly 0.45 μm diameter (Fig. 4 depicts the typical constitution of mycoplasma).
- It is observed that in the agar, mycoplasmas grow very slowly and it takes around a week to visualize their colonies. This growth rate is observed even under optimum growth conditions. Of note, the generation time of mycoplasma is between 1 and 9 h.
- Mycoplasmas gain the ability to readily infect a mammalian cell culture because of the following basic characteristics.

The basic characters of mycoplasma(s) are as follows:

1. Every eukaryotic cell, whether primary or secondary cultured, could be infected by mycoplasma.

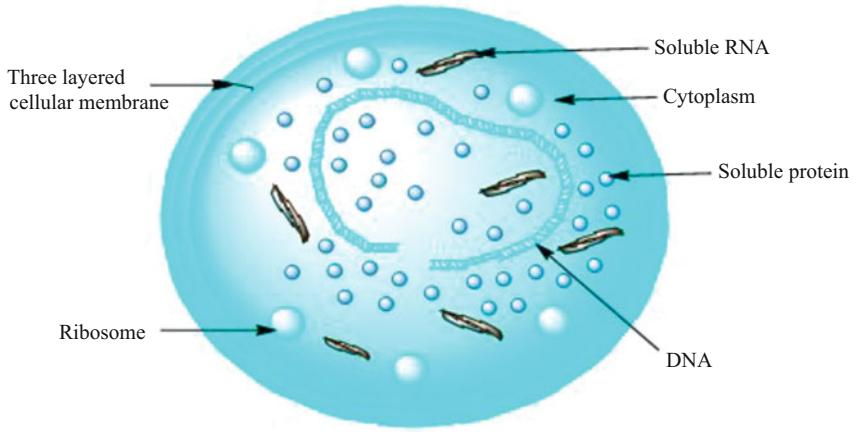

Fig. 4 Typical representation of a Mycoplasma cell. The bacterium is the causative agent of sexually transmitted diseases, pneumonia, atypical pneumonia, and multiple respiratory disorders. The cellular growth herein remains unaffected by many antibiotics

2. Microscopically, it has been observed that several mycoplasmas, sometimes even to the counts of 100, have an ability to bind on the surface of a single mammalian cell.
3. Mycoplasmas are the ***smallest self-replicating organisms*** having a diameter within 0.2–0.8 μm and a size of ~100 nm. Because of this small size mycoplasmas easily pass through the membrane filters. So, filter sterilization of mycoplasmas is not possible.
4. Thus, mycoplasmas are highly infectious to mammalian cells, some strains of which cause diseased conditions in human beings.
5. They lack the cell wall and, therefore, can't be destroyed by the bacterial cell wall damaging antibiotics such as penicillin.
6. They are fastidious in their growth requirements.
7. Thus, major precautions need to be exercised to prevent cell-culture contamination (Hayflick 1965; MacPherson 1966; Stanbridge 1971; Rottem et al. 2012).
8. Table 4 describes the basic differences between the growth and culture profiles of bacteria and mycoplasma(s).

4.4.1 Common Contaminating Mycoplasma Species

- Mycoplasmas mainly infect birds, bovines, experimental animals, and humans. So, all these living creatures can be sources of mycoplasma contamination among the various mammalian cell cultures.
- At present, the number of contaminated mycoplasma species isolated from the contaminated cell culture stands more than 20. While some of these species are pathogenic to humans, others are nonpathogenic.
- It is now well recognized that six mycoplasma species account for ~95% of all detected mycoplasma contaminations in mammalian cell cultures. These

Table 4 Distinctions in bacteria and mycoplasmas growth and culturing profiles

Bacteria	Mycoplasmas
Unicellular, prokaryotic, microscopic microorganisms	A genus of bacteria has the smallest size in the living kingdom. Smallest genome in the biological world
In general, bacteria contain a cell wall that surrounds the cell membrane	Mycoplasmas do not contain any cell wall
Bacteria have defined shapes, which could be *Coccus* (spherical), *Bacillus* (rod-shaped) or *Spirillum* (tubular)	Due to the lack of cell walls, mycoplasma does not have a definite shape. Mostly prevail as spherical or filamentous
Gram-positive or Gram-negative	Mycoplasmas are Gram-negative
Could be aerobic, anaerobic, or facultative aerobic	Either aerobic or facultatively aerobic
β-Lactam antibiotics such as penicillin can inhibit cell wall synthesis by preventing cross-linking of the small peptidoglycan layer	Since there is no cell wall, β-lactam antibiotics are of no use to control mycoplasma contaminations. Some other antibiotics such as minocycline, plasmocin can be useful

mycoplasmas are ***M. orale*****,** ***M. arginini*****,** ***M. fermentans*****,** ***M. hyorhinis*****,** ***M. hominis,*** **and** ***A. laidlawii*****.**

- The most common mycoplasma species infecting the oral cavity of a human being is ***M. orale***. Some estimations claimed that **20–40%** of all mammalian cell culture infections originated from the ***M. orale*** infected oral cavity of laboratory personnel.
- Other mycoplasmas that could contaminate a mammalian cell culture from the oral cavity of the laboratory personnel (due to mouth pipetting) are ***M.*** **fermentans,** ***M.*** **salivarium,** ***and M. pirum***.
- Erstwhile studies claimed that ***M. fermentans*** **and** ***M. hominis*** are the normal human microbial flora in the oropharynx and may contaminate the mammalian cell culture.
- It is estimated that FBS or FCS are the sources of **one-third** of all mycoplasma infections. ***M. arginini, M.*** **hyorhinis,** ***and A.*** **laidlawii** are mainly contaminated through serum used for mammalian cell culture.
- Trypsin solutions provided by swine are a major source of ***M. hyorhinis***. This species is a common inhabitant of the swine nasal cavity (Clyde 1964).

4.4.2 Sources of Mycoplasma Contamination

- It is evident from the above common contaminating mycoplasma species that the sources of mycoplasma infections are the **oral cavity of the laboratory personnel, solutions, and buffers used for cell culture, FBS, or FCS, trypsins from the swine, or even from the cultured cell source, and so on.**
- Previously contaminated mammalian cells may generate aerosols during handling that could further contaminate the cell culture medium and laboratory instruments, the typical sources of mycoplasma contamination.

- Several studies claimed that mycoplasma contamination through the oral cavity of laboratory personnel is the single largest source of its infection (McGarrity 1976).
- However, while FBS or FCS accounts for 25–40% of mycoplasma contamination, murine species account for only 0.5–1% of mycoplasma contamination (Rottem and Barile 1993).
- However, over the decade, mycoplasma contamination from the FBS and FCS gradually decreased because of several preventive steps including the development of mycoplasma detection assays.
- While primary cultured mammalian cells may be as contaminated as that secondary cultured mammalian cells, a comparative infection analysis shows that the chances of contamination in the primary culture may be less. The exact reasons for this observation are not known. Most possibly one of the reasons may be that cells that originated from a mammalian body are mostly germ-free whereas those from secondary culture have already undergone the culture process multiple times, giving rise to a greater contamination risk (Rottem and Barile 1993; McGarrity et al. 1985).

4.4.3 Contamination Rate of Mycoplasma

- Some estimations claimed that the mycoplasma contamination rate may be 10–85%.
- On average, the minimum contamination rate in the primary culture is as low as 1% and for continuous cell culture, it can be within 15–35%.
- The small size and lack of a cell wall in mycoplasmas allow their growth in very high densities (***10^7–10^9 colony forming units/mL***) in a cell culture medium.

4.4.4 Detection of Mycoplasma Contamination

- While mycoplasmas grow at a very high rate in a mammalian cell culture medium, their presence does not show any visible indications of contamination such as turbidity, pH changes, or even cytopathic effects.
- The average size of mycoplasma is less than 1 μm, rendering it invisible to the naked eye. It is very much difficult to detect mycoplasmas through a microscope until the attainment of extremely **high densities** that cause a cell culture to deteriorate. Till this stage, usually, there are no visible signs of infection.
- Even careful microscopic observation of live cell cultures cannot detect mycoplasma. The difficulty in being removed from the cultures as well as cumbersome microscopic visualization (owing to a very small size) makes mycoplasmas resemble viruses that are also very difficult to thoroughly eliminate from serum using membrane filtration.
- Quite persistently, there are 100–1000 mycoplasmas sticking to each infected mammalian cell. Primary cell cultures and cultures in the early passage are reported to be less frequently contaminated (typical order of 1% and 5%) than continuous cell lines (the typical order ranging within 15–35%.
- In addition, the fastidious growth requirements of mycoplasmas (unfortunately, easily provided for by cell culture medium) make them very difficult to grow and

detect using standard microbiological cultivation methods. These attributes make mycoplasmas the most threatening, ubiquitous, and devastating culture contaminants.

- A common hurdle in detecting mycoplasma contamination of microbial cell culture is its late identification, owing to which contaminating populating extents have already reached extremely high densities that result in significant cell culture deterioration.
- ***Direct culture of mycoplasma is the most common way to reliably detect its infection. It is still considered the gold standard of mycoplasma detection. However, in the practical sense, the direct culture of mycoplasmas is not suitable for most laboratory personnel because of the elaborate culture medium preparation regime, the prolonged incubation time, and the large sample volumes. Additionally, it is a qualitative technique and some of the mycoplasma species such as M. hyorhinis are hardly grown in culture, making them undetectable with culture-based methods.***
- The techniques used to detect the mycoplasmic contamination of mammalian cell cultures are fluorescent staining (e.g., Hoechst 33258), immunostaining, PCR, ELISA, autoradiography, or microbiological assays (Barile et al. 1962; Barile and Schmike 1963; Hopert et al. 1993; Drexler and Uphoff 2002).

Here is the list of major techniques used to detect mycoplasmas:

Histological Staining and Microscopy

Mycoplasma DNA can be stained by **DAPI (not permeable to the cell membrane and used for fixed cells)** or **Hoechst dyes (permeable to the membrane and used for live cells)**. Under the conditions of intense illumination, high magnification, and resolution, the photobleaching of Hoechst 33258 is used for the detection of mycoplasmas. Fluorescent staining is the best choice to detect Hoechst stain molecules. One of the negative points of this technique is the low signal provided by the DNA stain due to low mycoplasma count. In this case, the use of a highly sensitive camera may overcome the burden.

Transmission and Scanning Electron Microscopy

Noted as the prominent electron microscopic technique, scanning electron microscopy (SEM) scans only the surfaces of the mycoplasma. Another difficulty of the SEM is many types of cells have microvilli of comparable diameter and length. Thereby, SEM is inadequate to make a positive diagnosis in many cases. Under this situation, whole-mount electron microscopy is noted as the detection method for low-extent of mycoplasma contamination. Electron microscopic examinations of mycoplasma containing suspensions are frequently based on preparations made by negative staining.

Biochemical Methods

Several biochemical methods are used to detect the presence of mycoplasmas in the contaminated cell culture. Some of these methods are enzyme-based assays, gradient

electrophoresis separation of labeled RNA, and protein analysis. For example, mycoplasmas can be detected using an enzymatic mixture containing **biotin-dUTP** followed by indirect immunofluorescence. The uridine phosphorylase method can also be used to detect mycoplasma.

Immunological Procedures

Various immunological methods are used to detect mycoplasmas. In these methods, an antibody raised against the specific protein of mycoplasma is used to detect the mycoplasmas. The major use of mycoplasma-specific antibodies is fluorescence/enzymatic staining using antibodies and **ELISA. Autoradiography** may also be included in this category of mycoplasma detection methods.

RNA Hybridization

Contamination of mammalian cell cultures with mycoplasma can be detected with filter hybridization and liquid hybridization. The hybridization pattern of radio-labeled/fluorescent probe with mycoplasmal DNA is species-specific, enabling the identification of the characteristic mycoplasma species. The probes can detect the **rRNA, rDNA, or polymerase chain reaction amplification** products from these mycoplasma species.

Polymerase Chain Reaction

The best technique for screening mycoplasma contamination is the various types of polymerase chain reaction (PCR), including real-time PCR.

- The PCR technique for mycoplasma detection uses **16S rRNA** and **16S–23S rRNA** of different mycoplasma species. Suited modules rely on one- and two-step PCR (nested PCR), genus wherein species-specific and real-time PCR can be used to detect mycoplasmas. The main advantages of the PCR method in comparison with enzymatic and microbial techniques are higher sensitivity, specificity, accuracy, and prediction extent of positive and negative results. However, the presence of false-positive results is a major limiting factor of PCR-based methods, especially in nested PCR.
- The reliability of PCR detection is based on the manifested sensitivity, quality of the test sample, and primer specificity.
- Of all the various commercial kits used by pharmaceutical companies the most popular kit is ***EZ-PCR Mycoplasma Test Kit***. However, the kit needs to be standardized or optimized according to local conditions.
- To perform PCR different primer sets for the single, multiplexed, and nested regime is used that can detect a broad range of mycoplasma.

Controls for the PCR method:

- **Standard Positive Control:** Amplification of standard mycoplasma DNA with the help of standard mycoplasma-specific primers. It generates positive mycoplasma-specific product(s).

- **Standard Negative Control:** A sample of sterile water or growth medium, which must produce a negative result, i.e., no visible band on the PCR gel.

NB: Generally, every fortnightly to 3 months, mycoplasma detection is necessary.

4.4.5 Effects of Mycoplasma Contamination on the Mammalian Cells

The effects of mycoplasmas on the cultured mammalian cells depend upon several factors including the following:

- The cultural conditions include cultural medium composition.
- The experimental conditions.
- The type of cells being used.
- The intensity and duration of the contamination.
- The mycoplasma contaminations interfere with virtually every parameter measured in cell culture during routine cultivation or experimental investigations. In the worst-case scenario, the results of the complete experiment may have to be dumped and the experiments would have to be repeated.
- Mycoplasmas may retard the growth rate, in turn promoting the agglutination of suspension cell culture (Table 5).

  While some species of mycoplasmas may exert cytopathic effects including severe cytopathic effects on the cultured mammalian cells, some other mycobacterial species exhibit **very little overt cytopathology.**

Table 5 Mycoplasma detection methods with their sensitivity, advantages, and disadvantages

Method	Sensitivity	Advantages	Disadvantages
Direct DNA stain (e.g., Hoechst 33252)	Low	Rapid, cheap	Can be difficult to interpret
Indirect DNA stain (e.g., Hoechst 33258) with indicator cells (e.g., 3Ts)	High	Easy to interpret as the contamination is amplified	Indirect and thus more time-consuming
Broth and agar culture	High	Sensitive	Slow and may require expert interpretation
PCR	High	Rapid	Requires optimization
Nested PCR	High	Rapid	More sensitive than direct PCR, but more like to give false positive
ELISA	Moderate	Rapid	Limited range of species detected
Autoradiography	Moderate	Rapid	Can be difficult to interpret in the event of low-level contamination
Immunostaining	Moderate	Rapid	Can be difficult to interpret in the event of low-level contamination

- Mycoplasmas are extremely unfavorable to any cell culture: they cause **alteration in the host cells' growth characteristics, cell membrane composition, chromosomal structure,** etc.
- They also affect the host cells' **metabolism,** and **morphology** besides provoking **cytopathic responses,** rendering doubts over any data ascertained from contaminated cultures.
- The intensity of mycoplasma infective consequences with an increase in lasting time accompanied by decreased cell proliferation of cultured cells reduced saturation density and agglutination in suspension cultures.
- Notably, mycoplasma lipoproteins are potent activators of immune cells through their recognition by Toll-like receptor 2 (TLR2).
- Because of the virtually unlimited number of mycoplasma effects on cultured cells, only exclusive parameters have been listed here to just have a fair idea about the diversity of possible effects (Levine et al. 1968; Perez et al. 1972; Drexler and Uphoff 2002).

Here is a list of mycoplasma contamination effects on general mammalian cells and hybridoma cell culture:

4.4.6 Effects of Mycoplasma Contamination on Eukaryotic Cells

The contaminated mycoplasmas have several molecular and cellular effects on the mammalian cells culture of mammalian cells.

Here is a brief discussion about the effects of mycoplasmas on cultured mammalian cells:

Effects of Mycoplasmas Contamination on the Cellular Morphology of the Cultured Cells

Mycoplasmas change the expression of integrins and other cell surface proteins on the cultured cells. This alteration leads to changes in the morphology of the host mammalian cells.

Effects of Mycoplasmas Contamination on Genetic Materials of the Cultured Cells

- Mycoplasmas affect the synthesis of DNA **(replication),** RNA (transcription), and proteins (**translation)**.
- It causes the induction of chromosomal aberrations (numerical and structural alterations).
- Several reports indicate that mycoplasmas degrade the DNA of the host cells.

Effects of Mycoplasmic Contamination on the Metabolism of the Cultured Cells

- Mycoplasmas affect cellular metabolism.
- They can degrade simple sugars to get energy.
- Some mycobacteria use arginine deaminase to obtain energy. Therefore, mycoplasmas affect the arginine content of the mammalian cell culture medium.

4.4.7 Effects of Mycoplasmas Contamination on the Cell Signaling in the Host Mammalian Cells

Several cell signaling molecules of the cultured cells and their signaling events are affected by the contaminating mycoplasmas. These include activation or inhibition of lymphocyte activation, cytokine expression, viral propagation, transformation, altered signal transduction, etc.

4.4.8 Effects of Mycoplasma Contamination on Hybridomas Culture

- Hybridomas are hybrid cells produced by the fusion of plasma cells (having antibodies against single epitopes) with myeloma cells. The cultured hybridoma cells produce **monoclonal antibodies against single epitopes**.
- This technology was discovered in 1975 by Georges Kohler and Cesar Milstein who received the 1984 Nobel Prize in Physiology and Medicine.
- In hybridoma technology, single cells are cultured per cell culture container for the production of monoclonal antibodies.

However, contamination with the mycoplasmas affects the following:

- Cell fusion inhibition.
- Screening of the monoclonal antibody production and yield optimization of the monoclonal antibodies.
- Accidental antibodies against mycoplasmas may also be generated.

Thus, regular inspection of mycoplasmic contamination in the mammalian cell culture is essential and in case of its confirmation in mammalian cell culture, it must be eliminated by the following methods.

4.4.9 Elimination of Mycoplasmas from the Mammalian Cell Culture

- Once mycoplasmic contamination is confirmed in mammalian cell culture, it must be eliminated before mycoplasma contaminates everything related to cell culture in the laboratory.
- Autoclaving of mycoplasma containing medium, culture containers, followed by thorough cleaning of all glasswares and use of suitable disinfectants to clean every surface of the laboratory including those of various instruments, is the best way to get rid of mycoplasma.
- However, sometimes it is not feasible to trash the contaminated cultured cells, medium, and other materials because of the high cost and efforts involved in their culturing. Moreover, sometimes additional cells or media may not be available to repeat the same experiment. Under these conditions, it is required to clean the cultured mammalian cells from mycoplasmic contamination.
- The use of suitable antibiotics such as **quinolones, tetracycline, and macrolides** in the cell culture medium is the best way to eliminate the contaminating mycoplasma.

Table 6 Effective antimycoplasma antibiotics

Brand name	Generic name	Antibiotic category
BM-Cyclin	Tomalin	Macrolide
Solodyn	Minocycline	Tetracycline
Cipro bay	Ciprofloxacin	Quinolone
Baytril	Enrofloxacin	Quinolone
Zagam	Sparfloxacin	Quinolone
MRA	Not known	Quinolone

- Other agents which could be used against mycoplasmic contamination include **Myco-1 (tiamulin), Myco-2 (minocycline), Myco-3 (ciprofloxacin), and Myco-4.**
- The significance of Myco-4 is its broad spectrum of activity against the microorganisms including ***mycoplasma, acholeplasma, spiroplasma,*** **and** ***entomoplasma***) combined with very low cytotoxicity and a low resistance risk due to an initial biophysical mode of action (Schmitt and Daubener 1988; Drexler et al. 1994) (Table 6 describes various antimycoplasmic antibiotics/agents).

Ahead is the list of some standardized mycoplasma elimination methods from the mammalian cell culture.

4.4.10 Physical Procedures to Eliminate Mycoplasmas

- Autoclaving is the best procedure to eliminate mycoplasma. However, it is only used to destroy the cells/cell culture medium since autoclaves annihilate everything including temperature-sensitive materials of the culture medium.
- Other physical methods used to remove mycoplasmas include heat treatment and induction of chromosomal or cell membrane damage via photosensitization and irradiation with UV light.
- Because of the small size of the mycoplasma, filter sterilization may not be useful against mycoplasma contamination.

4.4.11 Chemical Procedures to Eliminate Mycoplasmas

Several chemical methods are used to eliminate mycoplasma. The most widely used of these methods involves the treatment of cells with detergents (e.g., **MycoZap**). Some methods that are included in this category comprise the washings of contaminated cells with ether and chloroform mixture, treatment with methyl glycine buffer, incubation with sodium polyanethol sulfonate, and culture in the presence of 6-methylpurine deoxyriboside.

4.4.12 Immunological Procedures to Eliminate Mycoplasmas

Various immunological methods are used to eliminate mycoplasmas. Administration of antisera against specific mycoplasmic proteins is the most preferred choice among these. Other immunological methods include the administration of complement in the cell culture medium, cocultivation with macrophages, and passage through nude mice.

4.4.13 Chemotherapeutic Procedures to Eliminate Mycoplasmas

A large number of laboratories use various antibiotics to destroy mycoplasma in the culture medium. On the other hand, mycoplasmas may be cultured in soft agar in presence of antibiotics. A combination of antibiotics and hyper-immune sera or cocultivation with macrophages may also be used (Table 6).

4.4.14 Prevention of Mycoplasmas Contamination at the Cell Culture Facility

A safe, clean and aseptic culture procedure must be followed at the cell culture facility. This includes the use of changing HEPA filters at the proper time, cleaning the laminar flow hood with 70% alcohol, cleaning all other instruments at regular intervals, discarding the cell culture trash in proper containers and eliminating them at the proper time, cleaning the floor, sinks, faucets and water bath from time to time and ensuring the restricted entry inside the cell culture laboratory.

- No eating, drinking, or smoking should be permissible within the laboratory.
- Use of sanitizers, sterilizers, and disposable gloves must be encouraged.
- Talking should be as much less as possible inside the laboratory.
- Late-night working should be avoided in the laboratory.
- Deep freezers and low-temperature facilities must be well-equipped for preserving the microbial cultures.
- No loud music or discussions should be permitted inside the laboratory.

4.4.15 Mycoplasmas Contamination Prevention at the Cell Culture Procedures

While it is not possible to eliminate the chances of contamination into the mammalian cell culture by any organism including mycoplasmas, there is sufficient room to reduce the chances of contamination. This includes the following:

- Cells should be collected from a reputable company such as ATCC, USA, to minimize the contamination.
- The complete cell culture medium including serum and other constituents must be tested for contamination before large-scale use or use for any other kind of experiment.
- A strict aseptic technique must be followed at every step of cell culture, as discussed in the above paragraphs.
- Standard mycoplasma procedures must be developed and authenticated in every laboratory so that in times of need these tests could be used to check the mycoplasmic contamination.

4.4.16 Mycoplasmas Contamination Prevention to Cell Culture Personnel

It is necessary to maintain the sterility of the cultured cells so that one's time, energy, and money should not be wasted besides a stringent need to take care of the health and physiology of the cell culture personnel and other people working in

the laboratory. This is because several mycoplasma species are pathogenic to the human body. As already discussed in the earlier paragraphs of this section of the book chapter, the following precautionary steps should be taken to prevent mycoplasmic contamination of the cell culturists.

- No eating, drinking, or smoking in the laboratory.
- No mouth pipetting in the cell culture laboratory.
- No unnecessary talking or trafficking within the cell culture laboratory premises.
- Jewelry may be taken off, long hair may be tied in the back.
- No pouring of cell culture medium containing cells.
- Handling only one cell line at a time.
- Nails should be cut for everyone handling and monitoring microbial cultures.
- ***McGarrity*** described various methods of prevention, control, and elimination of mycoplasma infection from the cell culture laboratory (McGarrity 1976; McGarrity et al. 2012).

4.5 Viral Contamination

- Viruses are microscopic infectious agents that take over the host cells' machinery to reproduce.
- Outside the host cell, these infectious agents do not show any sign of life. They are just like anything ordinary form of solid matter till they lie outside a host body.
- Each virus has a genetic material such as either DNA or RNA which is covered by an envelope (made up of proteins).
- Different viruses attack separate host cells. The viruses which use bacteria as host cells are called **bacteriophages**.
- The viruses which attack plant cells and use them as host cells are called **plant viruses**.
- The viruses attacking animal cells and subsequently using them as host cells are called **animal viruses**.
- Since viruses are host and tissue-restricted for their contamination, no cross-contamination is observed in their infections. For example, plant viruses do not infect human cells.
- Thus, only animal/human viruses may contaminate the mammalian cells in culture.
- Viruses are the smallest infecting agents (even smaller than mycoplasmas), and therefore, it is very difficult to detect viral contamination of the mammalian cell culture.
- Viruses are **intracellular obligatory parasites** and go for either **the lytic** or **lysogenic cycle**. While the **cytopathic lytic** effects of some viruses are detectable by a microscope, the viruses undergoing the lysogenic cycle get their genome integrated with the host's genome. Therefore, no visible effects of viral contamination have been observed to date. However, if a contaminated virus infects the

laboratory personnel's body, depending upon the virus, it may cause serious disease conditions.

- Several viruses that infect the human cells in the culture medium are ***human papillomavirus (HPV) hepatitis A, B, C, D viruses, retroviruses (*e.g.*, HIV1 and HIV2, Human T-cell lymphotropic virus,* etc.*), and herpes simplex virus (HSV), type 1 and type 2.***
- ***Polyomavirus*** *infects mouse pancreatic epithelial cells.*
- The mouse epithelial pancreatic cell lines have been reported of being contaminated by polyomavirus from which these cells were isolated.
- ***Simian Virus 40 (SV40***) infects the human kidney epithelial cells (Fig. 5).
- A common instance of viral contamination includes the ***adeno-associated virus (AAV),*** wherein assistance of a helper virus is needed by the provirus for replication.
- Viruses like ***lactate dehydrogenase virus (LDV)*** usually get diluted subsequently during passaging, thereby getting eliminated from the cell line(s).
- Thus, ultra-safety cautions must be preserved while working with viral cultures. In case of sudden mishappening, institutional environmental safety officers should be consulted regarding the safety procedures for working with potentially hazardous tissues, cultures, or viruses (Love and Wildy 1963; Mahi et al. 1991; Merten 2002; ATCC Website for virus contamination).

4.5.1 Detection of Viral Contamination

Detection of viral contamination of the mammalian cell culture is a difficult job because of the lack of effective measures or procedures to do so.

However, most of the tests used to detect various microorganisms contamination in mammalian cell culture are also applicable for viral detection. This includes **electron microscopy, ELISA assays or immunostaining, and PCR with appropriate viral primers.**

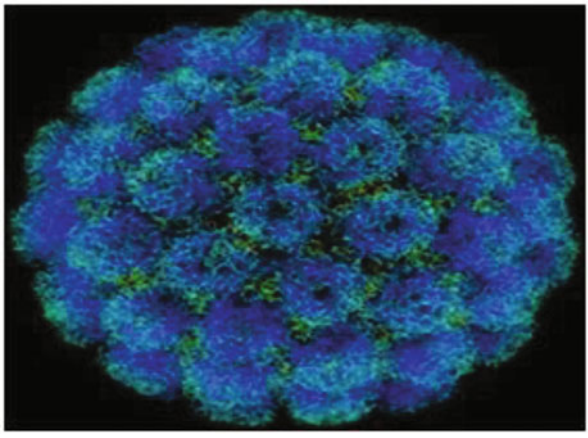

Fig. 5 Three-dimensional view of a Simian Virus 40. (Image reproduced from: https://www.slideshare.net/YashasviKumarSingh/sv-40)

PCR and ELISA for the Detection of Virus

Various types of PCR or reverse transcriptase PCR are used to amplify the virus-specific DNA in the contaminated culture. ELISA is used to detect virus-specific protein with the help of labeled antibodies against that protein. Both PCR and ELISA are the most widely used methods to detect viruses in contaminated mammalian cell culture(s). The same antibody may also be used for virus-specific immunocytochemistry or indirect immunofluorescent assays.

Electron Microscopic Observation of Viruses

- Early studies claimed that the virus belonging to *Arenaviridae* family was first identified by electron microscopy (**EM**) and then characterized as ***lymphocytic choriomeningitis virus*** **(LCMV**), an arenavirus transmitted by rodents as per the IFA assay and PCR.
- Thin section and negative stain **EM** examination are significantly instrumental in identifying viruses in growing cultures. For instance, the ***Filoviridae*** **(ebolavirus),** ***Monkey pox virus*****,** ***Bunyaviridae.***

Reverse Transcriptase Test of Viruses

- The **RTase** (**reverse transcriptase**) test detects all viruses having reverse transcriptase enzyme.
- In recent years, ultrasensitive RT tests have been developed. These tests are based on the product-enhanced reverse transcriptase (**PERT**) assays and are swiftly gaining eminence, having a 10^6–10^7 times lower detection limit than the conventional RT tests.

Immunohistochemistry for Virus Detection

- The ***West Nile virus (WNV)*** is a member of the *Flaviviridae* family. Genus Flavivirus was identified through implicit immunohistochemistry in the United States.
- Viral contamination can be screened and compared against normal tissues using IHC.
- ***NB: As indicated in previous paragraphs, utmost care should be taken while working on virus-infected mammalian cells since infection to the laboratory personnel may create serious diseased conditions*** (Hay et al. 1980; Ng et al. 2014).

4.6 Protozoan Contamination

Protozoa are a group of single-cell, microscopic animal cells that belong to the kingdom ***Protista.***

The major examples in this group are **amoebas, flagellates, ciliates, sporozoans, and many other forms** (Fig. 6 describes the general morphology of an amoeba).

As cell culture contaminants, both parasitic and free-living protozoa are implicated.

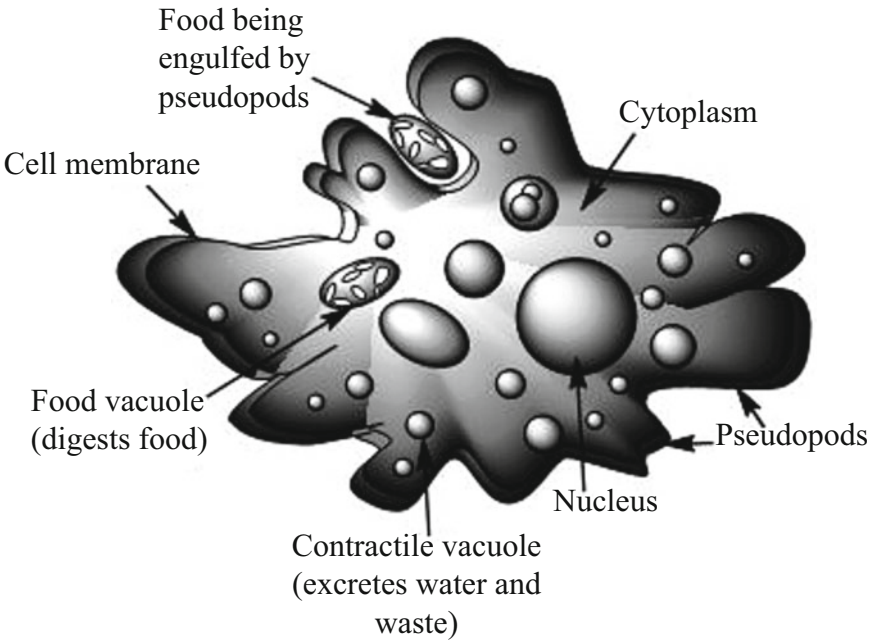

Fig. 6 Typical representation of amoeba morphology

- The major source of protozoa is the spores of amoebas present in the air or within the tissues (like nose, throat, etc.) and swabs of laboratory personnel infected with protozoa.
- It is observed that the following contamination to the mammalian cell culture, an amoeba takes around 10 days to show cytopathic effects (just like viruses) and destroy the cultured cells.
- However, because of the slow growth rate and morphological similarities of protozoa (particularly amoebas) with the cultured cells, it is somewhat difficult to detect the protozoan cells in culture, particularly at the initial stages of the culture unless concretely suspected as contaminants.
- Generally, protozoan contamination of the mammalian cell culture is rare as compared to other microorganisms.
- It is generally recommended to autoclave the protozoa-contaminated culture (Jahnes et al. 1957; Holmgren 1973).

4.7 Cross-Contamination by Other Cell Cultures

- Cross-contamination with unwanted mammalian cell lines is a major problem in mammalian cell culture. Most of the cross-contamination appears due to accidental coculture (Routray et al. 2016).

- With the discovery of karyotyping and various biochemical, and molecular techniques, it is now possible to accurately identify and characterize various mammalian cells. Now, it can be confirmed that a section of mammalian cell lines is already contaminated with other mammalian cell lines such as ***HeLa cells***.

  NB: HeLa cells are one of the fastest-growing cervical cancer cells, isolated from an African American woman named Henrietta Lacks.
- It was ***Gartler*** and his associates in 1966 who used **isoenzyme analysis** to show that 20 commonly used human cell lines were intraspecies contaminated by **HeLa cells** (Gartler 1967, 1968).
- In 1976, 18-monthly analyses of the 246 cell line test results revealed ~30% of cell lines as incorrectly designated:
- In 1981, a survey of mammalian cultured cells showed over 60 cell lines as HeLa cells, 16 human cell lines contaminated by non-HeLa human cell lines, and 12 erstwhile cases of interspecies contamination.
- Very soon, it was demonstrated that like HeLa cells, many other cells also can contaminate and grow in presence of other cells.
- In 2010, ***Capes Davis*** in their publication mentioned a list of cross-contaminated cell lines (Capes-Davis et al. 2010).
- As of December 2016, the database of misidentified or cross-contaminated cell lines maintained by the International Cell Line Authentication Committee (ICLAC) (iclac.org/databases/cross-contaminations) listed as many as 488 cell lines that are cross-contaminated or misidentified.

  NB: Authentication of the cultured cells can be done through the following procedures.

4.7.1 Ascertaining Cross-Contamination of a Cell Line with Other Mammalian Cells

The following characterization techniques are recommended for monitoring cell line identity. Most laboratories are equipped with at least one of these methods as part of their monitoring program:

Chromosomal Analysis/Karyotyping

It is a relatively simple method that involves preparing a metaphase spread with chromosome bands. Thereafter, the painting reveals the modal chromosome number and the presence of any unique marker chromosomes. The method can be applied to any tissue, ranging from amniotic fluid, blood, bone marrow, and those originating from the placenta. To examine amniotic fluid, amniocentesis is done whereas for the bone marrow, the bone marrow biopsy is done. Figure 7 outlines the general steps in chromosomal karyotyping.

Isoenzyme Analysis

- In 1960, this method was first proposed by Gartler, demonstrating the HeLa cells cross-contaminating the human cell lines.
- In this method, the isoelectric separation of a specific set of intracellular enzymes can be used to distinguish between the cell lines.

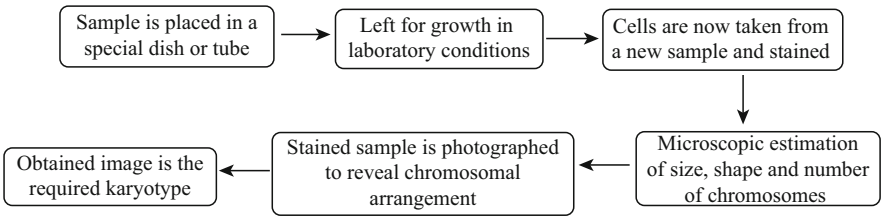

Fig. 7 Steps involved in karyotype analysis: microscopic estimation and imaging of chromosomal size, shape, and number

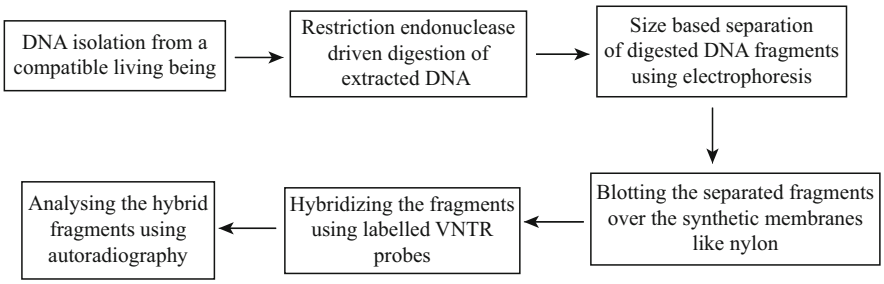

Fig. 8 Typical steps of DNA fingerprinting technique

- In general, at least three enzymes are used for the determination of the isoenzyme gel electrophoresis banding pattern.
- Some examples of these types of enzymes are Glucose-6-Phosphate Dehydrogenase (**G6PD**), Lactate Dehydrogenase (**LDH**), Malate Dehydrogenase (**MD**), and G-Protein-Coupled Receptors (**GPCR**).

Immunological or Biochemical Techniques

These methods enable the detection of markers that are unique to tissue, cell line, or the species from which the working culture is derived.

DNA Fingerprinting Analysis

DNA fingerprinting measures variation in length within minisatellite DNA containing variable numbers of tandem repeat sequences (**TRS**) to detect both intra- and interspecies contamination. Figure 8 pinpoints the chronological steps in DNA fingerprint analysis. Major applications of DNA fingerprinting concern forensic and paternity investigation, where it accounts for implicit identification. Other than these areas, DNA fingerprinting is also used to ascertain the frequency of a particular gene in a population which attributes to diversity. In this manner, the changed frequency of one or more genes can be used to determine its evolutionary significance.

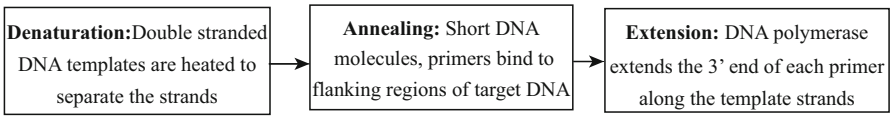

Fig. 9 Typical steps in a polymerase chain reaction (PCR)

Polymerase Chain Reaction Analysis

Polymerase chain reaction (**PCR**) enables the amplification of specific genes or regions of a gene. Figure 9 illustrates the steps in a typical PCR (one chain), distinguished as denaturation, annealing, and extension.

In the denaturation, double-stranded DNA templates are heated to get them separated. In the subsequent step of annealing, short DNA molecules called primers bind to the flanking regions of the target DNA. Thereafter, DNA polymerase acts on the 3′ end of each primer strand to complete the multiplication of source DNA. The three steps are repeated 25–35 times to exponentially generate the exact copies of the target DNA.

DNA Barcode Regions Evaluation

- DNA barcoding is one of the most important recent techniques that distinctly characterizes a particular species based on the sequence of a standard short section of DNA in its genome.
- Generally, instead of evaluating the sequence of a certain region of a particular genomic DNA, DNA fragments from the **mitochondrial gene cytochrome C oxidase subunit I** can be used for DNA barcoding.
- The sequence obtained by this technique should ideally match the **DNA barcode library** from a species.

Short Tandem Repeat Profiling

- By definition, short tandem repeats (STRs) are specific regions or locations on the chromosomes **(nuclear DNA)** that contain a short sequence core that repeats itself within the DNA molecule. This short repeated region of the DNA is called **microsatellite DNA**.
- The STR profile serves as a **helpful marker** for the identification of a particular cell type and confirms whether a given cell line is contaminated with any other cell line.
- At present, STR is recognized as a "***current international reference standard***" to confirm the identity of a particular cell.
- In STR, the variations in the length of the repetitive DNA sequences within the microsatellite DNA are determined which are subsequently compared with the standard data already available from the previous works of various scientists.
- Three steps are involved in STR profiling, namely, **amplification, electrophoresis, and interpretation**.

NB: At present, STR profiling can be commercially done. At present, STR profiling is a must to confirm the identity, purity, and suitability of the cell substrate for manufacturing use.

- Here is a list of publications that explained various tests to identify the cross-contamination of mammalian cell culture with other unwanted cells (Stulburg et al. 1976; Moore 1992; Nelson-Rees et al. 1981; Buehring et al. 2004; O'Donoghue et al. 2011; Almeida et al. 2016).

5 The Effect of Microbial Contamination on Cell Lines

The experimental mammalian cell culture research and biomanufacturing industries involving culture contamination pose serious problems and challenges including complete waste of cell culture materials to the serious challenges to human health.

Here is a list of consequences of contamination in mammalian cell culture:

5.1 Effects on Adherence of the Cultured Cells

Most mammalian cells are adherent in nature (except certain blood cells). This adhesion is possible due to the surface of mammalian cells as well as the expression of various proteins and residual charges on the surface (plasma membrane) of the mammalian cells. This adhesion is highly required for the growth and proliferation of mammalian cells. Cell culture contaminations may alter the expression of adhesion molecules and integrins, leading to the cell's detachment from the culture containers.

5.2 Effects on Growth and Proliferation of the Cultured Cells

The mammalian cell culture medium is very rich in nutrition. The proliferation and doubling rates of contaminating microorganisms are much faster than those of mammalian cells. For example, a bacterium just takes 20–30 min to divide. Due to the rapid growth rate of microorganisms and accumulation of toxic metabolites, the medium pH changes. This severely affects the growth and proliferation of the cultured mammalian cell(s).

5.3 Effects on Metabolism of the Cultured Cells

It is an experimental observation that microbial contamination severely affects the metabolism of cultured mammalian cells. As a result of this, carbohydrate and lipid metabolism may be severely affected, leading to an alteration of the plasma membrane. **Metabolomic** analysis of the cultured mammalian cells reveals **mycoplasmic** contamination which could, in turn, drastically affect the cellular metabolism of the cultured mammalian cells.

5.4 Effects on DNA, RNA, and Protein Synthesis

The synthesis of DNA **(replication),** RNA **(transcription),** and protein **(translation)** may be affected by contamination of the mammalian cell culture.

5.5 Effects on Apoptosis of the Cultured Cells

Several levels of contamination-related alterations, including changes in medium pH and accumulation of toxic metabolites by the contaminated microbial cells in the mammalian cell culture medium, may activate the programmed cell death **(apoptosis) of** the cultured cells. This leads to the death of the cultured cells.

5.6 Cytopathic Effects on the Cultured Cells by Viral Contamination

Viral contamination also exhibits **cytopathic effects** on the cultured cells. While in the **lytic phase,** a virus ruptures the host cells. In the **lysogenic phase,** the viral genetic material is integrated with the host cell genome and is subsequently propagated to further generations as a part and parcel of the host cell genome. This integration of the viral genome inside the host cell DNA may result in **mutations leading to cancer and other diseased states** (Lincoln and Gabridge 1998).

NB: Technically, it is not possible to completely eliminate the chances of microbial contamination into the mammalian cell culture. However, it is possible to reduce the contamination probability as much as possible by having a detailed knowledge of contamination sources.

6 General Procedures to Prevent or Minimize a Cell Culture Microbial Contamination

To prevent or at least minimize the contamination of a mammalian cell culture medium, one should follow the overleaf instructions:

1. Wear Gloves and Lab Coats
2. Work Only in a Biosafety Cabinet/Laminar Flow Hood
3. Scientifically Use the Laminar Flow Hood
4. Work On One Cell Line at a Time
5. Minimize Nonsterile Environment Exposure of Cells
6. Add Antibiotics and Antimycotics Only if Necessary
7. Spray Everything with 70% Ethanol
8. Buy Sterile Reagents and Keep Them Sterile
9. Use Sterile Labware

10. Use Autoclaved Filter Tips and Change Them Often
11. Check Your Cells Periodically
12. Follow Good Lab Practices
13. Clean Your Incubator and Water Bath Regularly
14. Decontaminate a Laminar Flow Hood by Fumigation
15. Routinely Clean the Cell Culture Room
16. Proper Disposal of Biohazards
17. Use Common Sense

Here is a brief discussion of the above instructions:

6.1 Wear Gloves and Lab Coats

- Using gloves and lab coats reduces the chances of contamination.
- It is recommended to use lab coats only inside your cell culture laboratory or there may be separate lab coats for the cell culture laboratory.
- Clean the lab coats often.
- Sometimes, facial masks may be necessary to reduce the chances of contamination.
- Rinse the hands with 70% alcohol before and after working.

6.2 Work Only in a Biosafety Cabinet/Laminar Flow Hood

- All human, primate, or mammalian cell lines obtained from an outside source (e.g., repositories such as the American Type Culture Collection (**ATCC**), other institutions, or investigators, must be treated using biosafety levels instructed by that source.
- Prepare the complete cell culture medium inside the laminar flow hood only, filter sterilize and make aliquots of as small a volume as possible and store them at 4 °C.
- Before every use, the cell culture medium should be warmed at 37 °C. Thereafter, the outer surface of the bottle should be rinsed with 70% alcohol.
- This will prevent unnecessary contamination from the water bath.
- Open and close the cell culture vessels only inside the laminar flow hood.

6.3 Scientific Use of the Laminar Flow Hood

To ensure a sterile environment inside the laminar flow hood, one must use it scientifically and do the following:

- Before the use of the laminar flow, the laminar flow inside the hood should be switched on and its floor should be cleaned with 70% alcohol.

- The air outlet and inlet of the laminar flow hood should be blocked free to allow an unhindered air passage.
- In general, work should be done well inside the laminar flow hood, not at the edges.
- Most users prefer not to keep waste disposal container(s) inside the hood and rather keep it outside. Keeping the waste disposal material or reagents for a long time inside the hood can increase the contamination risk.
- Finally, after the day's work, clean the hood floor with 70% alcohol.
- Check the life of the laminar flow filter and replace it as and when necessary.
- Fumigate the laminar flow area as and when it is necessary.

NB: There are numerous safety hazards associated with UV light exposure, such as cornea burns and skin cancer. (See, Biosafety Technical Bulletin: Ultraviolet Lights in Biological Safety Cabinets, https://ncifrederick.cancer.gov/ehs/ibc/Media/Documents/UVLights.pdf.) For this reason, the NIH, CDC, NSF/ANSI, and the American Biological Safety Association all agree that ultraviolet (UV) lamps are not recommended, nor are they necessary.

6.4 Work on One Cell Line at a Time

Cross-contamination by other mammalian cell lines is a major problem in mammalian cell culture. As mentioned earlier for example previously, it was shown that HeLa cells can easily contaminate a large number of other mammalian cells apart from easier accommodation in presence of other cells. To minimize such happening, one should work on one cell line at a time.

6.5 Minimizing the Exposure of Cells to Nonsterile Environments

Since so much effort is invested to maintain a sterile environment inside the incubator and the hood, it must be made sure that cells spend the least time outside these sterile environments.

- For example, while viewing the cells under a microscope or while transferring them from the incubator to the hood.
- Sometimes people work on some techniques (e.g., time-lapse imaging) which require the cultured cells to be kept for a long time outside the incubator. Following the work completion, if one wants to incubate the cells again in a CO_2 incubator, the cells should be put in a separate incubator, to minimize the contamination chances.

6.6 Add Antibiotics and Antimycotics Only if It Is Necessary

- Pipette the medium into the cell culture vessels and centrifuge tubes instead of pouring it. This will reduce the chances of contamination.
- Add antibiotics and antimycotics to the complete medium at a proper concentration as and when required.
- Antibiotics are substances produced by one group of microorganism and acts against another group of microorganism at a low concentration.
- Antibiotics are specifically utilized to prevent or control bacterial growth. Similarly, antimycotics are utilized to prevent fungal growth (molds/yeast, etc.).
- Some prefer not to regularly use antibiotics and antimycotics as this practice may generate drug-resistant strains.
- In **serum-free medium,** either less or preferably **no antibiotics** and **antimycotics** should be used.

  NB: Various serum proteins interact with antibiotics and antimycotics and decrease their chance of directly interacting with the human cells. However, in a serum-free medium, in absence of serum proteins, antibiotics and antimycotics may directly interact with the cultured mammalian cells and may affect them.
- Bacterial cell wall interfering antibiotics such as penicillin cannot be used against mycoplasmas because a mycoplasma does not have a cell wall.

6.7 Spray Everything with 70% Ethanol

- Take 70 mL anhydrous or dehydrated alcohol; add 30 mL distilled water to make 70% alcohol. Water increases the efficacy of ethanol in killing bacteria and some viruses.
- The hand gloves, the outer surface of cell culture media bottles, and every other single material that may serve as a source of contamination must be cleaned with 70% alcohol.

  NB: Labware should be marked with ethanol-proof markers.

6.8 Buy Sterile Reagents and Keep Them Sterile

- For cell culture, every reagent used must be sterile.
- This encompasses cell culture medium, PBS, or any other product that could come into cellular contact.
- If a laboratory worker uses reagents for other work that is not sterile, such as antibodies, he/she should not use them for the cell culture.
- It is necessary to distinctly and separately store the stocks of sterile and nonsterile reagents with proper labeling.
- After adding all the culture medium components, including serum and other additives, the medium must be filtered and sterilized.

- Every single reagent including culture medium can be aliquoted aseptically in small containers inside the laminar flow hood before storing in a clean environment.
- Similarly, one can also buy prealiquoted and previously sterile reagents.

6.9 Sterile Labware Should Be Used

- The labware must be autoclaved before use.
- All the consumables related to the cell culture must be sterile.
- Use only sterile culture containers (Petri plates/flasks), pipette tips, centrifuge tubes, and microfuge, and only open them aseptically inside the laminar flow hood,
- Seal the additional/extra containers inside the laminar flow hoods before taking them out.
- Use only filtered, sterile water inside the laminar flow hood or for any other works related to cell culture.

6.10 Filtered Tips Should Be Used

- The use of filter tips in the mammalian cell culture reduces the chances of contamination.
- The tips prevent the solution to reach or touch the pipette.
- Therefore, chances of contamination should be reduced, particularly from the pipettes.
- Change of filter tips frequently further reduces the risk of contamination.
- Importantly, if one wants to pipette something out of a bottle, avoid using pipettes to prevent direct contact with the inner periphery of the bottle.
- Instead, use a pipette gun with longer pipettes that reach deeper inside the bottle.

6.11 Check Your Cells Often

- It is recommended to check the cells often by using an inverted microscope.
- The medium color, turbidity, morphology, and growth pattern of cells in floating versus adherent patterns should also be checked.
- It is necessary to learn and distinguish between movable cell debris and contaminants such as bacteria while observing a culture under inverted/phase contrast microscopes.
- If one suspects that his/her sample is contaminated with mycoplasma, available commercial kits could be used.

6.12 Follow Good Labeling Practices

- Good records of one's cells, supplements, and media stocks could help in prompt identification of contamination sources.
- Similarly, if one knows the batch of medium or supplements that were being used, he/she can examine or discard the affected stock to avoid repeated contamination.
- Good labeling can also prevent contamination of one's experimented cells with others: every single person doing experiments should make sure that the cells in use for the experiments are only those exclusive ones for which the experiments were planned. This is a huge problem in mammalian cell culture studies, with as many as 30,000 studies using the wrong cell lines.
- Thus, it is highly pertinent to make sure that cells are labeled properly with no inadvertent cross-contamination risk among different samples.

6.13 Clean Your Incubator and Water Bath Regularly

Most of the new incubators have self-cleaning capacities, others must be cleaned manually. While doing that often, the protocol will vary depending on the incubator type and functioning.

- Occasionally, once a month, fumigate the CO_2 incubator so airborne microorganisms are thoroughly eliminated.
- Water trays are used inside the CO_2 incubator to maintain moisture. Every week the tray must be cleaned, autoclaved, and refilled with fresh autoclaved water. In general, chemicals, antibiotics, or antimycotics are not used to prevent microbial growth in the water tray.
- Similarly, the water in the water bath where we warm up the cell culture medium and other solutions should be regularly changed.
- Make way for a water bath treatment each time it is replaced.

6.14 Decontamination of Laminar Flow Hood by Fumigation

While formaldehyde remains in the gaseous form its 40% aqueous solution is called formalin.

Formaldehyde is highly toxic to microorganisms.

- For sterilization of the CO_2 incubator and the whole cell culture laboratory, formaldehyde vapor is used. However, for fumigation, formaldehyde is used in combination with potassium permanganate (a strong oxidizing agent).
- Add 35 mL formalin (37% or 40% formaldehyde) to 10 g potassium permanganate/m^3 in a porcelain or steel container. Immediately exit the room. Do not wait for the reaction to begin. Thereafter, the room should be closed overnight.

6.15 Routinely Clean the Cell Culture Room

- Routinely, clean the tissue culture room and always keep the room dust-free so that microorganisms cannot adhere to the dust particles.
- Every instrument should be cleaned with 70% alcohol.
- Fumigation of the whole cell culture laboratory is necessary if lots of aerosol generation is suspected during cell culture handling or fungal spore prevalence in the air.

6.16 Carry Out a Quality Control of Cells

Carry out quality control of cells to monitor the prevalence of likely contaminating pathogens regularly or whenever necessary.

Conduct regular sterility inspection of the culture medium and associated reagents.

6.17 Disposal of Biohazards Properly

- Biohazardous materials should be kept in a separate labeled container.
- To decontaminate such wastes, treat them with 10% bleach (hypochlorite), trigone of clorox. These agents are strong oxidants, destroying the enzymes and other proteins of the contaminated microorganisms.
- Following treatment, the biohazard materials may be disposed of at a dedicated place, from where these wastes would be taken care of using the waste disposal management of the Institute/University.

NB: In the event of contamination, it is necessary to inform all other lab personnel who are working in the same cell culture laboratory and may use the CO_2 incubator. This will help them to check their cells and if necessary, suitable steps could be taken to prevent contamination or decontaminate (for the cells already contaminated).

6.18 Use Common Sense

Common sense is indeed highly crucial to preventing contamination.

- A mammalian cell culture laboratory must be dedicated only to culturing mammalian cells. Thus, work on microorganisms such as fungi or bacteria should not be allowed in this kind of laboratory.
- Do not talk while working on cell culture.
- Do not work if you are ill.
- Close the incubator door as soon as work is completed.

- Wash your hands before and after work on cell culture.
- Do not disrupt the net and clean environment of the cell culture laboratory.

7 Handling Unwanted Contamination in the Culture Flask

- First and foremost, confirm unwanted contamination by monitoring the turbidity, medium pH, and color using microscopy and other techniques as discussed in the previous paragraphs.
- Remove all the contaminated culture flasks/Petri plates and store them in a place where no cell culture experiments are being conducted.
- Wipe out the exterior surface of the noncontaminated flasks with 2.5% sodium hypochlorite, and then with 70% ethanol. Incubate them in a separate CO_2 incubator without using any other culture containers from the remaining lab personnel. Allow them to grow and check from time to time, for a possible contamination event. If a visible sign of contamination is observed like changes in turbidity, medium color, etc. these flasks mandate a discarding.
- For contaminated culture, containers add 10% sodium hypochlorite and leave for 2 h before rinsing down the sink with a sufficient amount of water.
- Thoroughly cleaning the CO_2 incubator is a necessity to prevent future contamination.
- The water tray should be cleaned with 2.5% sodium hypochlorite, which is thoroughly autoclaved and can be reused, as and when necessary.
- Clean the cabinets with 70% alcohol and 2.5% sodium hypochlorite, after soaking for 5 min.

  Nowadays programmable CO_2 incubators are available which increase the temperature of the incubators, acting like a hot oven to kill all the germs.
- After all the cleaning works are completed, gowns should be put for laundry and fresh gowns should be used for further cell culture, in the same incubator.

7.1 Recovery and Monitoring

- Monitoring the contamination is a prerequisite to checking the recovery of the cell culture medium.
- For complete recovery, it is necessary to identify the specific species and strain of the contaminated microorganisms before taking suitable actions appropriately.
- In case of inability to identify the contaminating microorganisms due to lack of resources or any other reasons, help from a diagnostic laboratory may be helpful.

7.2 Persistent Contamination

- Sometimes contamination persists despite taking all the steps necessary to work in a neat and clean environment. Persistent contamination signifies observation of

one or more contamination resulting outcomes every week while working on mammalian cell culture. Under this condition the following steps should be taken:

- You may have to discard all the culture flasks.
- You have to discard the already opened culture medium.
- You may have to discard the antibiotics and antimycotics.

NB: Start the cell culture afresh using new culture flasks, a new culture medium, and new cell stock in a completely clean and decontaminated CO_2 incubator.

7.3 Training

All the laboratory personnel involved in the mammalian cell culture must be trained by senior laboratory personnel before commencing the work on cell culture. In this case, every single step necessary to maintain the sterility of the mammalian cell culture must be followed. Finally, building the capacity to work in a neat and clean environment should be ensured to working in a mammalian cell culture laboratory.

7.4 Laboratory Layout

The layout of a laboratory could be so developed which reduces the risk of contamination via placing the cell culture cabinets out of main thoroughfares, placing waste disposal collection containers away from clean work areas, and sterile media storage. There is a concomitant dire need of segregating any work with the preservation of microorganisms for preparing clean cell cultures. For instance, the placement of class II cabinets should be carefully monitored to avoid interference by laboratory furniture/equipment, walls, benching, doors, and other cabinets.

7.5 Cleaning and Maintenance

Laboratory cleaning is the foremost preventive measure, wherever contamination is concerned. A formal routine cleaning regime for the general laboratory premises will indeed minimize the chances of environmental contamination, thereby enabling a significant reduction in day-to-day contamination risks.

7.6 Routine Quality Control

The establishment of a cell banking regime capable of providing a stock of low passage cultures subjected to repeated quality control screening is yet another primitive aspect of good cell culture practice. Periodic testing of cell lines in laboratory use is likely to enhance confidence over their aseptic status and sudden chances of contamination. Quite often, threatening mycoplasma does not fall

unrecognized and where they do arise, are dealt with in a significantly quick manner so that the additive influence on any simultaneously initiated task is minimized.

NB: Here is a list of publications that describes the prevention or elimination of microbial contamination from mammalian cell culture (McGarrity and Coriell 1971; Wolf 1979; Crueger 1990; Mather and Roberts 1998; Ryan 2008).

8 Conclusions

Contamination either by microbes or other cells is a major challenge to the success of mammalian cell culture. The unwanted presence of microorganisms and mammalian cells other than the cells of interest are the major contaminating agents in cultured mammalian cells. Out of all contaminating microorganisms, while bacteria and fungus (yeast/molds) can be easily detected by the naked eye, contamination with the mycoplasmas and viruses is hard to detect. Contamination with the protozoans is generally very rare and therefore does not pose any major problems. However, contamination with unwanted mammalian cells such as HeLa cells is a common problem in mammalian cell culture, and statistics suggest that as high as 30% of various mammalian cells and cells line stored by various International Cell Repositories such as ATCC are contaminated. Specific tests are available to confirm the contamination by every microorganism or by mammalian cells. While it is generally accepted that contaminated cells must be discarded, sometimes because of various reasons such as rare cell lines or scarcity of desired cells force a researcher to decontaminate the cultured cells. This chapter discusses the various types of contaminations, their detections, and eliminations from the cultured mammalian cells.

9 Cross-References

► Troubleshooting of Mammalian Cell Culture

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Mammalian Cell Culture Types and Guidelines of Their Maintenance

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_6-2

Abstract

Mammalian cell culture can be classified into three types, namely, primary, secondary, and continuous cultures. The maintenance and growth of cells isolated directly from the parental mammalian tissues/organs in cultures comprise the **primary cell cultures**. When the primary cultured cells are further subcultured (**propagated**) or **passaged** in new culture vessels, it is called **secondary culture**. Since secondary cultured cells are derived from the primary cultured cells, they are also called **extensions** or **continuations** of primary culture. The normal mammalian cells have a finite ability to undergo divisions in physiological conditions, and that is why these cells are **mortal**. So, primary and secondary cultured cells originate from the normal mortal cells having a capacity of limited divisions. However, in certain pathophysiological conditions such as **cancer,** few cells can be originated within a mammalian body with a capacity to divide **uninterruptedly** or **continuously** as long as they are supplied with proper nutrition and an ideal growth environment. Because of the **continuous** or **infinite** division capacity, this type of cell culture is also called **continuous cell culture**. Additionally, mammalian cells possess a slightly **negative charge** on their plasma membrane and require **adherence** with the cultured vessels for their growth or proliferation. While most mammalian cells are **adherent in nature**, some blood cells (e.g., B/T lymphocytes) are **nonadherent**. This chapter narrates primary, secondary, and continuous cell culture, as well as adherent and non-adherent cell culture procedures.

Keywords

Normal/ Mortal Cells · Immortal/ Cancer cells · Adherent Cells · Nonadherent Cells/ Cells in Suspension · Primary Culture · 2ndary/ Extended Culture · Continuous Culture · Adherent Culture · Suspended Culture · Cryopreservation of Cells · Thawing of Cells

1 Introduction

This chapter describes the various types of mammalian cell cultures. Based on the origin or source of cells, mammalian cell cultures can be broadly divided into three groups. They are (1) **primary culture**, (2) **secondary or extended culture**, and (3) **infinite or continuous culture**. For primary culture, cells are directly isolated from the **tissues or organs before being** in vitro cultured. When primary cultured cells are further propagated into a larger number of new culture containers, it is called secondary culture. This indicates that the secondary culture of cells is nothing but the extension of primary culture in new cell culture containers. ***The main purpose of secondary culture is to get more cells for experimental or other purposes*** (Ratafia 1987; Paul 1975).

Both primary and secondary cultured cells originate from normal tissue or organs and have **a limited life span** because the maximum cell division capacity of any normal cell in a mammalian body is around **50–100** only. Thus, primary and secondary cultured cells originate from normal and physiologically healthy tissues or organs, and are also called **mortal cells, exhibiting finite cell divisions** (Willmer 1960). When any normal cell is **transformed** due to **spontaneous (e.g., DNA replication error/error in DNA repair, etc.) or induced mutation (e.g., effects of biological agents such as viruses or radiations or chemical agents**), it results in the formation of **immortal cells. These immortal cells** have the capacity for **continuous or infinite division and therefore they are also established as immortal cell lines**. **Epigenetic modifications (e.g., DNA acetylation/methylation)** also give rise to continuous cell divisions. Transformation of a normal mortal cell to an immortal cell occurs either in the mammalian body itself or experimentally in the laboratory as and when a normal cell is treated with a virus or chemical agents, etc.

Additionally, mammalian cells carry **a net negative charge on their surface**. Based on the interaction and **anchorage or attachment** of cells with the culture vessels (Petri plates/flasks, etc.), mammalian cells can be divided into **adherent** and **nonadherent or suspension** cells**.** While an adherent cell mandates attachment with the cell culture containers for their growth, a nonadherent cell does not need to **attach or anchor** with the cell culture container and thus grows as **suspension culture** (Birch and Arathoon 1990). While most mammalian cells are adherent in nature, few blood cells such as **B/T lymphocytes are nonadherent** and therefore grow as a suspension culture. Thus, cultured cells can be classified based on either the **origin of cells** or **anchorage dependence**. The present chapter describes the above classification of mammalian cells based on their origin and anchorage dependence.

2 Classification of Mammalian Cell Culture

Based on cellular origin, mammalian cell culture is broadly divided into the following three types:

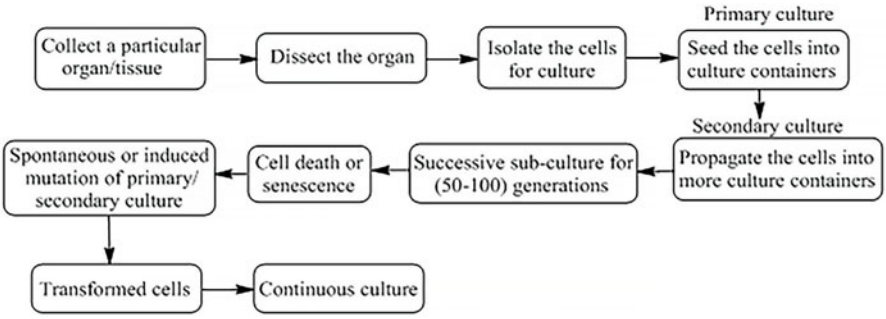

Fig. 1 Schematic representation of primary, secondary, and continuous mammalian cell cultures, typically distinguishing the handling populations at an instant

Primary culture of mammalian cells
Secondary culture of mammalian cells
Continuous culture of mammalian cells

Figure 1 depicts the three variations of mammalian cell cultures. Here is the discussion of these culture types.

3 Primary Culture of Mammalian Cells

The maintenance and growth of cells isolated directly from the parental mammalian tissues/organ (such as kidneys, blood vessels, etc.) comprise the primary cell cultures (Dieter F et al, Hülser 2006).

The primary culture has the following steps:

Collection of tissues/organs
Isolation of cells
Centrifugation to collect the cells
Seeding and culture of the primary cells

The procedure of collection of tissues/organs, followed by isolation and culture of various mammalian cells is discussed in chapters ▶ "Isolation and Primary Culture of Various Mammalian Cells"; ▶ "Primary Culture of Immunological Cells"; ▶ "Culture of Neuron and Glia Cells"; ▶ "Culture of Continuous Cell Lines"; and ▶ "Stem Cell Culture and Its Applications." Very briefly, organs/tissues will be collected either from euthanized experimental animals or human organs discarded during organ transplantation, aborted fetus, or accidentally dead individuals, etc. The organs will be aseptically treated with enzymatic digestion, mechanical dissociation, and other procedures to get the isolated cells (Freshney 1994).

Now, the following paragraphs directly discuss the primary culture of mammalian cells.

3.1 Procedure of Primary Culture of Mammalian Cells

- The cell suspension collected from the tissues/organs needs to be put (**seed**) into a cell culture container and requires incubation in a CO_2 incubator.
- **Seeding** simply means to spread a defined amount **(number of cells in a specific medium volume)** of a **cell suspension** into a flask or a Petri plate (**called cell culture vessels**).
- However, since most mammalian cells are adherent in nature **with a slightly negative charge on the surface**, the cell culture containers **need to be treated with various adhesive agents such as collagen, gelatin, laminin, fibronectin, poly-L-lysine, etc.**
- A brief discussion of the adhesive agents and their applications is already discussed in chapter 4.

Here is a brief discussion of the coating of the culture vessels with 0.1% gelatin.

3.2 Coating Cell Culture Vessels with 0.1% Gelatin Solution

Gelatin, a partially hydrolyzed collagen, is the cheapest mammalian cell adhesive agent. **Gelatin** possesses the **RGD sequence** of collagen, making it highly effective for **cell adhesion**. Of note, the tripeptide **Arg-Gly-Asp** (**RGD**) consists of Arginine, Glycine, and Aspartate. It was originally identified as the amino acid sequence within the extracellular matrix **(ECM)** protein fibronectin that mediates cell attachment. So, gelatin is generally used for mammalian cell attachment with the cell culture containers. Figure 2 depicts the chemical structure of gelatin, wherein distributed hydrophilic and hydrophobic sensitivities present the suitability of forbidding any random aggregation, making the interactions receptive with plasma membrane lipids.

Here is the procedure for coating cell culture containers with gelatin:

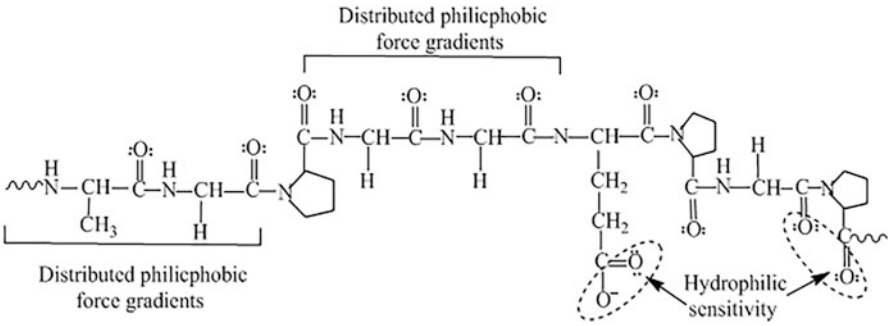

Fig. 2 Chemical structure of gelatin, depicting the characteristic philicphobic force gradients for its aggregation-forbidding abilities

- Aseptically in the laminar flow hood, pour 5–10 ml of sterile 0.1% gelatin solution into the culture vessels to cover the entire surface of culture plates and flasks.
- Incubate gelatin solution containing vessels at 37 °C incubator for 1 h.
- Take the cell culture vessels once again to the laminar flow hood.
- Discard excess gelatin solution.
- Rinse the culture vessels with 1× Dulbecco's Phosphate Buffer Saline (**DPBS**).
- Without drying the gelatin-coated vessels immediately, add the culture medium and use them.
- Alternatively, add 5–10 ml DPBS.

3.3 Primary Culture of Mammalian Cells

- Store at 4 °C until use (generally use within a week. Long-duration storage may cause contamination (Fig. 3)).

NB: The major importance of primary cultured cells is that these cells are isolated and collected directly or straight from the tissue/organ and are, therefore, cultured* in vitro *for the first time.

3.4 Salient Features and Benefits of Primary Cell Culture

- Primary cultured cells have **no prior passage** or **propagation.**
- Cells in primary culture best resemble the natural tissue.
- When cells are isolated for primary culture from a tissue or organ, it may be **a mixture of various cells, representing the composition of that particular tissue or organ.**
- An appropriate laboratory procedure must be used **to select the desired cell type from the mixture** (described in chapter 7), **subsequently enriching that particular desired cell type.**

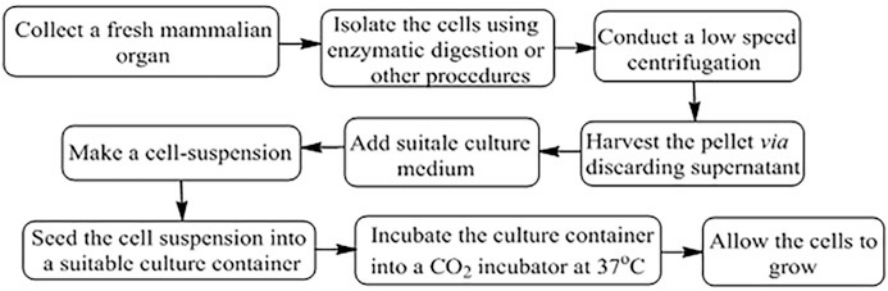

Fig. 3 Schematic representation of mammalian cells isolation and culture procedures

- Cells with desired properties can be selected using specific growth conditions such as medium.
- To get an increased number of cells, the primary cultured cells are harvested from the cultured containers and seeded into more culture containers, and further subcultured.
- This transfer of cells from one to many culture containers is called **passaging** or **propagation** or **subculturing** (Geraghty et al. 1990).
- With each subsequent subculture, the cell population becomes **more homogenous** as the faster-growing cells predominate.
- Subculturing of primary cells leads to the **generation of cell lines**. **A cell line has at least one passage**.
- Cell lines can passage several times before they become senescent/aging. For example, endothelial cells can be subcultured up to 50 passaging.
- The lineage of cells originating from the primary culture is called **a cell strain** (Geraghty et al. 1990).
- Primary cells have a finite life span. **This means all the primary cells originating from a normal healthy tissue/organ will die after a certain number of divisions.** As described previously, for example, endothelial cells perish after around 50 passages.
- However, if the source tissue/organ from where the cells are isolated contains tumor/cancer cells, they will grow indefinitely, provided they have a constant supply of nutrients as well as oxygen.
- Cells such as **neurons** do not divide in vitro or even in vivo, so these cannot be **used as primary cultures**. However, supporting cells present in the nervous system such as neuroglial cells can divide.
- Similarly, a mature human RBC does not contain any nucleus and therefore no question of cell division.
- The pattern of growth in primary cultured cells may be either **adherent** or **suspension**, which is discussed in the later section of this chapter.
- Even after overnight incubation in a CO_2 incubator, if the adherent cells float or do not attach to the cell culture containers, they may have either been damaged severely during isolation or could be in apoptotic or pre-apoptotic stages. Under this condition, the chances of cell growth are least. Generally, apoptotic cells look **round** when observed in an **inverted microscope**.
- For primary culture, there needs to be a large number of cells than secondary cultured cells because many of the primary cultured cells die even before growth due to various stresses like mechanical agitation or handling, the effect of proteolytic enzymes, etc.
- If the cells attached/adhere to the culture container grow at a slower pace than expected, the number of live cells in the cell culture container is less than expected. In this situation, **cell-to-cell cross-talk is not appropriate because of the low cell population in the cell culture containers**. **Consequently**, cells either grow slowly or do not grow at all.

NB: Mammalian cells secrete various molecules in the cell culture medium which diffuse to the other cells and interact with them. This is called cell-to-cell cross-talk and is viciously involved in active cell division.

- Alternatively, **inappropriate medium composition and culture conditions** may also be responsible for no or less growth of the cells (Riley et al. 1991).
- Thus, the choice of cell culture medium with proper additives is very important for proper culture and growth of primary cells. For example, vascular endothelial cells such as human umbilical vein endothelial cells (**HUVECs**) grow better in the presence of vascular endothelial growth factors (**VEGF**) (Hartung et al. 2002; Langeler et al. 1993).
- As discussed in chapter 4, the cell culture medium pH is around 7.4–7.5 (the pH of human blood). The cell culture medium pH and contamination are monitored using ***phenol red*** addition to the cell culture medium. If the medium color (phenol red) changed overnight, there may be contamination with microorganisms including bacteria. While acidic pH (<7) develops a yellowish medium, a basic pH manifests as a pink color medium.
- Every single step of isolation and mammalian cells culture must be performed aseptically, inside the laminar flow hood only, minimizing the chances of contamination (Werner et al. 1992; Ryan 1994).
- The most popular primary cells used in research are ***epithelial cells, fibroblasts, keratinocytes, melanocytes, endothelial cells and muscle cells, and hematopoietic and mesenchymal stem cells*** (Geraghty et al. 2014).
- **Explant culture:** When a piece of tissue or organ is directly put into a cell culture container without disaggregating or dissociating cells for the culture purposes, this is called **explant culture**.
- While explant culture is the oldest culture used by Harrison, it is still in use by some section of scientists.

3.5 Benefits of Using Primary Cultured Cells

- Primary cultured cells exhibit significant resemblance with normal natural cells.
- With more passaging of cultured containers, the cells of primary culture may lose many of their natural characteristics. The reason for extensive usage of primary culture cells is because of reduced protein expression in latter passages due to **accelerated senescence**, alongside **spontaneous apoptosis**. For example, both angiotensin I-converting enzyme (**ACE**) and **prostacyclin** synthesis decrease as a function of the number of passages (Esquenet et al. 1997). Additionally, it is observed that in the primary culture of endothelial cells, estrogen receptors (**ERs**) gradually decrease after four to six passages (Portela et al. 2010). There are ample examples like these.

- ***The major benefits of primary culture of mammalian cells are as follows:***
  - Cells are isolated directly from the tissues and organs, and therefore, early passaged cells closely resemble in vivo conditions.
  - Immortalized cell lines cannot be the best representations of in vivo physiological conditions since these cell lines harbor many mutations in their genome (Peterson et al. 1973).
  - Primary cultured cells are utilized to understand the basic physiology of the cells including morphological and structural-functional relationships, developmental biology, cell-to-cell communications, and to understand various pathophysiological or diseased conditions including cancer, diabetes, Alzheimer's disease, and so on (Geraghty et al. 2014).

4 Secondary or Extended Culture of Mammalian Cells

When the cells of primary culture are further subcultured in new vessels, this culture is called **secondary culture**. Therefore, secondary cultured cells are derived from the primary cultured cells and are also called **extensions or continuations** of primary culture.

4.1 Propagation of Primary Cultured Cells to Secondary Culture

- **Propagation** of cells is also called **passaging or subculturing or splitting**. Propagation or passaging denotes the removal of cells from an old cell culture container (vessels) into more new cell culture containers containing freshly prepared medium (McGarrity et al. 1985).
- Passaging helps in further subculturing of cells into more cell culture containers.
- As the seeded cells for primary culture grow in cell culture containers, they use up the nutrients from the cell culture medium, produce toxic metabolites, as well as divide and slowly the culture containers become confluence. Under these conditions, these cells need to be split into an increased number of cell culture containers. For adherent cells, either cell scrappers or trypsin-EDTA may be utilized to detach the cells from the cell culture containers (Chang 1954; Holley 1975).
- Like primary culture, secondary culture also provides finite cell lines (if the source cells are not transformed or cancerous).
- The purpose of secondary culture is **to get more cells** that can be utilized for various purposes. As discussed in the primary culture section, if the secondary cultured cells originated from a normal healthy tissue/organ of primary culture cells, they divide a limited number of times. It is further worth noting that if the primary cultured cells originated from a tumor or cancer, the secondary cultured cells could be cultured continuously, provided nutritional medium and ideal culture conditions are maintained (Hayflick 1998).

NB: Splitting is the process by which one allows the cells to multiply through separation (splitting) into different culture flasks. For example, you grow one 75 mm flask of endothelial cells, and after 2–3 days, they are almost entirely confluent (80–90%) and then could be split into three 75 mm flasks.

4.2 Procedure of Secondary Culture of Mammalian Cells

The primary cultured cells grow as secondary cultures either as adherent cells or cells in suspension. Figure 4 depicts the stepwise procedure.

NB: In cell culture biology, confluence refers to the percentage of a culture dish surface covered by adherent cells.

- For trypsin-sensitive cells, instead of trypsin-EDTA, a **cell scraper** may be used.
- For **strictly adherent cells** following the addition of trypsin-EDTA, one may need to shake the cell culture container or incubate it at 37 °C for early detachment.
- For **loosely adherent cells**, shaking the cell culture container might be enough to detach the cells from the containers. There is no need of adding trypsin-EDTA.
- For cells growing as suspension culture, the treatment is done either with trypsin-EDTA, due to which cell removal by cell scraper is not necessary. So, directly one can go for the centrifugation step by transferring the cell suspension into the centrifuge tube (Table 1 describes salient characteristic differences between the primary and secondary culture of mammalian cells).

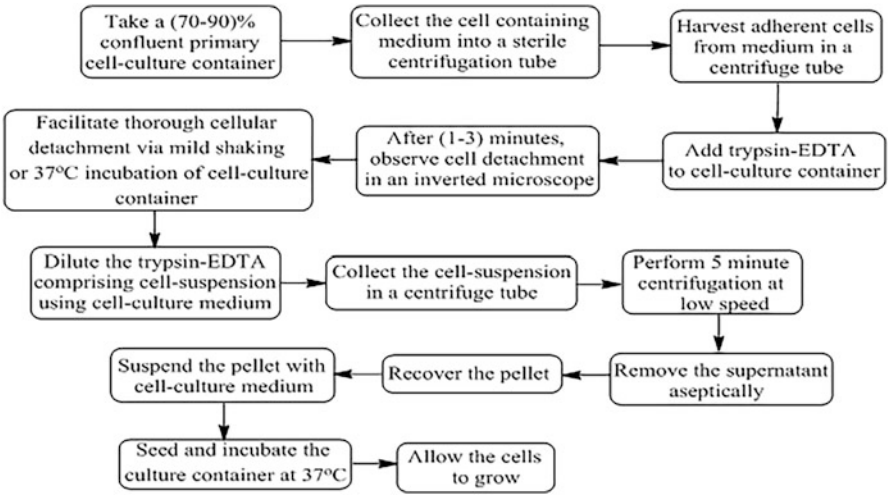

Fig. 4 Schematic representation of primary culture cells subculturing enabling formation of secondary culture cells. **The details of subculturing are described in the propagation of adherent and nonadherent cell culture section**

Table 1 Distinctions of primary and secondary mammalian cell cultures

Primary cell culture	Secondary cell culture
Mammalian tissues or organs are directly used to isolate the cells for primary culture	Originate from primary cell culture
Cultured for the first time	Derived from an existing culture
As these are directly taken from the tissues or organs and cultured for the first time, these exhibit at least some level of similarities under the in vivo conditions	Since these cells are cultured many times, there may be changes in several characteristics of the in vivo conditioned cells. For example, after a couple of passages, endothelial cells may gradually lose estrogen receptors due to lower expression. After around 15 passes, endothelial cells lose the capacity to express estrogen receptors
As these cells are cultured for the first time, the mutation rate is negligible or none	Could be cultured for many generations. High chances of mutations. The cells can even be converted into transformed cells
These cells have a definite life span. For example, after around 50 passages, endothelial cells die	Just like primary cultured cells, these cells also have a definite life span. However, if transformed may live indefinitely
The risk of contamination is high, particularly during isolation from the tissues or organs. More difficult to maintain	The risk of contamination is lower. Comparatively easy to maintain

5 Continuous Culture of Mammalian Cells

- In certain pathophysiological conditions such as cancer, few cells can be originated in the mammalian system (body) with a capacity to divide uninterruptedly or continuously as long as they are supplied with nutrition and an ideal environment for growth.
- Because of the **unlimited or infinite** capacity of divisions, continuous cells are also called **immortal or infinite cells.**
- Immortal cells can be passaged or propagated continuously as long as the proper environment (temperature, oxygen, pH, humidity, etc.) and the proper cell culture medium (nutrition) are provided.
- Thus, this kind of culture of immortal cells is called **Continuous Cell Culture**.
- The immortal continuous cell lines originated due to **spontaneous or induced mutation** either by radiations (such as ionizing radiations, ultraviolet rays, etc.), chemicals (such as benzene, arsenic or asbestos, etc.), or biological agents (such as *Human papillomavirus (**HPV**) causes cervical cancer* or *Helicobacter pyroli causes gastric cancer*) or because of epigenetic alterations (such as acetylation/methylation of DNA) (Chang-Liu and Woloschak 1997).
- The continuous immortal cells may give rise to **tumor/cancer cells/cancer stem cells**. An immortal continuous cell line can also be created in the laboratory with the help of certain types of virus called **oncovirus** (e.g., E6/E7 proteins from

Table 2 Culture and growth characteristics of finite and continuous cells

Finite cells	Continuous cells
Limited divisions (50–100)	Continuous/infinite divisions
Anchorage-dependent growth	Anchorage-independent growth
Cells grow as monolayers	Cells may grow as multilayers
Cells grow in low density	Cells grow in high density
Cells can be maintained cyclically	A steady-state culture is possible
Generally, the growth rate is slow	Usually, the growth rate is high
Low yield	High yield
Low cloning efficiency	High cloning efficiency
Control parameter: generation time, specific markers	Control parameter: strain characteristics
Cells exhibit contact inhibition	No contact inhibition is noticed
High serum requirement	Low serum requirement

HPV which causes cervical cancer) or treatment with chemicals such as arsenic which causes skin cancer.

- ***Cell lines transformed under laboratory conditions in presence of specific cancer-causing viruses or* in vitro *culture conditions give rise to continuous cell lines.***
- For example, **BEAS-2B cells** are human bronchial epithelial cells, transformed by **Simian virus 40 (SV40) T antigen**. Similarly, the **HEK293 cell line** is a permanent cell line established from **primary embryonic human kidney cells**, which were transformed with sheared human **adenovirus type 5 DNA**. The adenoviral genes expressed in this cell line allowed the cells to produce very high levels of recombinant proteins.
- Mutation of specific genes (**proto-oncogenes/tumor suppressor genes**) of these cells and alteration of **telomere** are some of the reasons for the conversion of normal cells with limited cell division capacity to immortal cells with indefinite or continuous cell division capacity. The following table describes the differences between finite and continuous or infinite cultures (Table 2).

5.1 Procedure of Continuous Mammalian Cell Culture

Continuous culture of various mammalian cells (including cancer cells) is described in detail in **chapter 10**.

6 Adherent and Nonadherent Cells

- In general, most mammalian cells maintain a negative charge on their surface. However, the overall charge distribution may vary from cell type to cell type, particularly across the biological membrane (plasma membrane). The presence of

phosphatidylserine and ***Gibbs-Donnan membrane equilibrium*** may be some reasons attributing to this, as suggested by various scientists.

- Negative charge confers adhesive traits to most of the cells, with mammalian cell culture grade **polystyrene-made culture vessels**.
- While most mammalian cells are adherent in nature, some of these are loosely adherent and even a few (e.g., B lymphocytes/T lymphocytes) are suspended in nature.
- While the adherent cells attach to the polystyrene-made culture containers, the nonadherent cells such as B/T lymphocytes remain suspended in the culture medium. Without attachment to the culture containers, adherent cells cannot grow.
- Experimentally, it is observed that adherent and free-floating cells can be tailored to the specific cell type by addition to the suitable cell culture plates and requisite growth factors.
- In a large-scale culture of mammalian cells in bioreactors, many of the adherent cells are adapted to grow as nonadherent cells.
- Based on the adherence or attachment or anchorage, cells can be divided into adherent and nonadherent categories.

6.1 Adherent Cells

Most mammalian cells require attachment for growth and are recognized as **anchorage-dependent cells**. The adherent cells are usually derived from tissues of organs such as kidneys where they are immobile and embedded in connective tissue. They grow while remaining adhered to the cell culture containers (Petri plates/flasks). The adherence is possible due **to the specific interaction of cell surface proteins with a distinctive charge of cell culture containers (e.g., polystyrene)** (Surachi 1999; Phelan 1996). While some of the cells are **firmly adherent (e.g., endothelial cells)**, several others are **loosely adherent (e.g., MCF-7 breast cancer cells)**.

The specific characteristics of the adherent cells are described as follows:

- Appropriate for most cell types, including primary cultures.
- Require periodic passaging or propagation, but is easily visualized under an inverted microscope.
- Generally, normal mortal cells form a single cell layer.
- Cells are dissociated enzymatically (e.g., trypsin) or mechanically using a cell scraper.
- Growth is limited by surface area, which may affect product yields.
- Require tissue-culture-treated containers/vessels.
- Used for cytology, continuous product harvesting, and several other research applications.

NB: Before seeding the firmly or strictly adherent cells (e.g., endothelial cells) into the polystyrene-coated cell culture containers, the culture containers must be treated with various cell adhesive agents.

- For treatment of the mammalian cells, culture containers/vessels with adhesive agents, **gelatin, collagen, fibronectin, laminin, poly-L lysine**, etc. are used. Gelatin is the **most common** and cheapest adhesive agent used in endothelial and other adhesive cell cultures.
- While collagen, fibronectin, laminin, etc. are **natural protein components** of mammalian extracellular matrix (**ECM**), gelatin is a derivative of **mammalian collagen**, a major ECM component, and poly-L-lysine is a **positively charged synthetic protein**. Poly-L-lysine was originally produced from **bacteria**.

6.2 Propagation of Adherent Cells

Experimentally, in the laboratory any cells (except some blood cells) whether primary/secondary or continuous cultured (immortal cell line) grow as adherent cells.

The propagation of the adherent cells involves the following steps:

1. Detachment of cells from primary cell culture containers
2. Dilution of proteolytic enzyme-treated cell suspension
3. Centrifugation of the diluted proteolytic enzyme-treated cell suspension
4. Discarding the proteolytic enzyme-containing supernatant and resuspension of pelleted cells
5. Seeding cells into adhesive agents treated cell culture containers
6. Incubation of cells in a co_2 incubator and finally growth of the cells

Here is the detailed procedure.

6.2.1 Detachment of Cells from Cell Culture Containers

Take one (80–90)% confluent cell culture container into the laminar flow hood and aseptically collect the culture medium into a centrifugation tube.

Adherent or loosely adhered cells are firmly or loosely attached, respectively, to the cell culture containers. So, the first step in the detachment of adherent cells from the culture containers involves the addition of proteolytic enzymes. The most commonly used enzyme is **trypsin-EDTA**. Individual cell-specific protocols are discussed in the respective chapters.

Subculturing of adherent cells commences via detachment from the culture vessel surface via enzymatic or mechanical treatment. Table 3 comprises various cell dissociation procedures.

NB: As observed from the above table, the following points are important for the dissociation of adherent cells from the cell culture containers:

- Loosely adhered cells are treated with **0.01%** trypsin to easily detach them from the monolayer.
- Strongly adhered cells in monolayer (one single layer) are treated with **0.25% trypsin**, termed as **trypsinization of cells**. Incubation of the cell culture container

Table 3 Applications and working mechanisms of various cell-dissociating agents

Applications	Dissociating agent (s)	Procedure
Loosely adherent cells	Gentle shaking or rocking of culture vessel	Shake-off
Cell lines are sensitive to proteases	Cell scraper	Scraping
Strongly adherent cells	Trypsin	Enzymatic dissociation
Highly dense cultured cells	Trypsin + collagenase	Enzymatic dissociation
One of the major applications is detaching epidermal cells as confluent, intact sheets from the surface of culture dishes without dissociating	Dispase	Enzymatic dissociation
A major application is for strongly adherent cells; a direct substitute for trypsin; applications require animal origin-free reagents	TrypLE™ dissociation enzyme	Enzymatic dissociation

into a 37 °C incubator may enhance the process of trypsinization. Examples: endothelial cells, macrophages, dendritic cells, epithelial cells, and some adherent cell lines (Darlington 2008).

- Highly dense cell cultures having multiple layers are treated with **trypsin plus collagenase** to break the intercellular collagen and form the single-cell suspension. **Example: fibroblast cells** (Zinninger and Little 1973).
- In case, the whole-cell monolayer harvests as confluent, intact sheets from the surface of culture dishes (without dissociating intercellular proteins) from the monolayer, **dispase** is used. **Example: epidermal cells.**
- Strongly adherent cells are treated with **trypsin** plus **shaking** the culture flask heavily and/or incubating at 37 °C following trypsin addition or **scraping** with a **cell scraper**. **Example**: endothelial cells like human umbilical vein endothelial cells (**HUVECs**).
- Sometimes, only EDTA solution is used for detaching and harvesting cells to avoid trypsin, as the cellular proteins should remain intact for a particular experimental design.

6.2.2 Exceptions

Sometimes, proteolytic enzymes are avoided for sensitive cells as these enzymes can digest the surface proteins on the sensitive cells (Yang et al. 2010).

- Similarly, EDTA solution is also not used where there is a risk of interference with intracellular **calcium and magnesium**-like minerals. People working on cell signaling such as calcium signaling might not prefer using EDTA.
- In these situations, the harvesting of cells is done using a mechanical process. This is called as **mechanical way of harvesting cells**. As, in the case of loosely adherent cells and mitotic cells, gentle up and down **pipetting** and/or gentle

shaking and rocking of the culture flask is done to dislodge the cells from the surface.
- **A cell scraper** is also used to remove the mammalian cells being used for **molecular biology research such as the isolation of DNA** or **RNA**.

6.3 Dilution of Proteolytic Enzyme-Treated Cells

- Following trypsin-EDTA addition, cells must be microscopically examined at periodic intervals (e.g., inverted microscope), for their physical state (detached and floating) in the culture medium.
- This is necessary because trypsin and other enzymes are proteolytic (**digest the cell surface proteins**) and therefore they may cause lysis of the cells if co-incubated for a long time.
- So, as soon as the enzyme-treated cells are detached (generally within 1–5 min), they are diluted by the cell culture medium. Cell culture medium and serum present in the medium contain various proteins that neutralize the proteolytic enzymes (**the medium and trypsin ratio may be 7:1 (vol./vo**l.)).
- Generally, the **used-up cell culture medium is collected before the cell culture is treated with proteolytic enzymes** and used for trypsin dilution.
- However, if the used-up medium contains a large number of dead cells, it must be discarded and a fresh medium without additives could be used for dilution of trypsin-treated cells.

6.4 Centrifugation of Diluted Proteolytic Enzyme-Treated Cells

The diluted cells are separated using centrifugation (segregate as pellet) at 1000–3000 rpm for 5–7 min, 4 °C.

6.5 Resuspension of Pelleted Cells After Discarding of Supernatant

- The supernatant comprising proteolytic enzyme mixed in cell culture medium is discarded before resuspending the cell pellet in fresh complete cell culture medium.
- The volume of medium for cell suspension depends on the number of cells and the size of the cell culture container.
- For example, working on a 100 mm Petri plate requires nearly 10 ml complete medium with at least $\mathbf{1 \times 10^5}$ **endothelial cells** is necessary for adequate cell growth.
- The cell pellet is suspended by up and down pipetting of the medium.

6.6 Seeding Cells into Adhesive Agents-Treated Cell Culture Containers

The suspended cell pellet is now seeded into cell culture containers. Since the cells are adhesive, the culture containers must be treated with various adhesive agents before seeding the cells.

6.7 Incubation of Cells in a CO_2 Incubator

The suspended cells are now incubated in a CO_2 incubator at 37 °C, 5% CO_2, and 95% moisture for further growth. The percentage of CO_2 may change depending upon the specific requirement of cultured cells.

NB: Generally, cells that are 60–70% confluent (early to mid-log phase cells) are used for subculture. This rule applies to adherent cells, nonadherent cells, and also for immortal cancer cells. For subculturing, fully confluent cells (100% confluent) either do not grow properly or grow very slowly possibly because of the cell to cell contact inhibition.

- Thus, for adherent cells, thoroughly confluent cells come in contact with each other and inhibit each other's growth, a practice known as **contact inhibition**.
- Such cell types may need time to grow after being subcultured.
- Some reports indicate completely confluent cells capable of some **differentiation**.
- Cancer cells exhibit less or no contact inhibition and therefore continually grow as **multilayered cells**. However, they generally possess a deteriorating rate of cell division after two doubling times in the same culture container.
- Even in cancer cells, continuous culture for several generations may change or mutate the genome of the cells. In general, ATCC recommends not to continuously culture the immortal cells after 15–20 generations.

6.8 Importance of Cell Adhesion

- Cell adhesion is highly essential for the attachment of adherent cells with the culture containers. The adherent cells cannot grow without attachment to the culture containers.
- The adhesive characteristic of the various mammalian cells is utilized for their isolation and purification. Examples are routine isolation of bone marrow-derived blood cells (e.g., macrophages), separation of adult stem and progenitor cells, and isolation and characterization of cancer stem cells, for example, colonospheres, mammospheres, and neurospheres. This aided in identifying and expanding the rare cancer stem cells (Briske-Anderson et al. 1997).

6.9 Limitations of Adhesive Cell Culture

Following are the limitations observed in adhesion-dependent isolation and purification of mammalian cells:

- The purity of recovered cells is low.
- Risk of cross-contamination with other adhesive cells.
- Cell adhesion is regulated by several proteins. So, the sorting of cells based on the adhesive properties of various proteins is based on a specific methodology adopted.
- Chances of false-positive results.

6.10 Nonadherent Cells

Prominent cell types growing in nonadherent regimes include stem cells, embryoid bodies, tumorspheres, B/T lymphocytes, etc. The cells naturally growing in suspension and those having lost their anchorage dependency can be separated from the adherent counterparts via culturing in ultralow attachment plates in the absence of serum. Desired cells either grow as **a single-cell suspension or aggregate** to form **floating spheroids**. Figure 5 distinguishes the cancer cells grown in adhesive and nonadhesive modes on the bone-marrow-derived mesenchymal stem cells.

Salient features of nonadherent cultures are as follows:

- Nonadherent cells are also called **anchorage-independent cells**. Since these cells grow as a suspension in the cell culture medium, they are also called **suspension cells**.
- In this type of **culture,** a single cell or **small aggregates of cells** multiply while **suspended** in an **agitated liquid medium**. Thus, it is also referred to as cell **suspension culture**.
- All suspension cultures **are derived from cells of the blood progenitor cell** (hematopoietic stem cells which are $CD34^{+}$) because in the in vivo conditions, these cells are suspended in plasma.

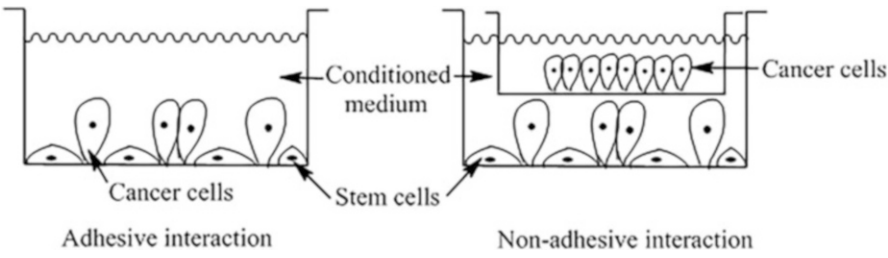

Fig. 5 Cancer cells grow in adhesive and nonadhesive interactive modes

- However, not all cells originating from **hematopoietic stem cells** can divide (**e.g., mature RBC**). These cells cannot grow as suspension cultures (e.g., **differentiated monocytes, which are also called macrophages**, grow as adherent cells).
- An important example of suspension culture is the culture of various lymphocytes **(B/T lymphocytes)**.
- For large-scale mammalian cell cultures, such as the industrial production of some recombinant protein or antivirus vaccine, the adherent cells are adjusted and propagated to nonadherent or suspension cells. For example, Chinese Hamster Ovary (**CHO**) cells that are cultured on a large scale using **bioreactors** are adjusted to culture as nonadherent cells. This not only increases the number of cells in a fixed medium volume but also the recombinant protein production by CHO cells.

6.11 Propagation of Nonadherent Cells

The nonadherent cells are passaged with a small amount of culture containing a few cells, diluted in a large volume of freshly prepared cell culture medium. These are distributed either in one large or several small culture containers. Alternatively, one can centrifuge the cell suspension and resuspend it in a larger volume on being distributed into multiple culture containers (Todaro et al. 1965). Table 4 summarizes the differences between adherent and nonadherent cell cultures (Ratafia 1987).

7 Changes in Mammalian Cell Culture Medium

Depending upon the **confluence, utilization of the medium, and the accumulation of toxic metabolites**, the old medium needs to be replaced by a freshly prepared new medium (Phelan 2007; Phelan and May 2015, 2016, 2017).

So, medium changes are necessary because of the following reasons:

- To add **fresh nutrition** to the actively dividing cells (nutrients will be rapidly utilized and depleted in actively dividing cells).
- To remove **apoptotic/necrotic** cells that may be **present/floating** in the culture medium.
- To remove **toxic metabolites** produced by the cells. Some metabolites such as **lactic acid** may decrease the pH of the culture medium, causing a serious deterioration of the health and physiology of cells (Conlon et al. 2001).
- To add **fresh antibiotics/antimycotics** being destroyed or degraded during culture medium incubation at 37 °C in a CO_2 incubator.

NB: Antibiotics** and **antimycotics are added to the culture medium to prevent bacterial or fungal growth, respectively.

Table 4 Comparative description of adherent and nonadherent cell cultures

Adherent cell culture	Nonadherent cell culture
Most mammalian cells including human cells are adherent in nature, e.g., endothelial cells, epithelial cells	Only a few blood cells (e.g., B cells and T cells) are nonadherent and grow in suspension
Require polystyrene-made plates. Additionally, the cell culture vessels may be treated with cell adhesive agents such as gelatin, fibronectin, laminin, poly-L-lysine, etc.	No need for treatment with any adhesive agent. However, some suspension cells may need shaking or stirring for adequate gaseous exchange
Once the culture vessels are confluent with the adherent cells, the culture containers are treated with enzymes (e.g., trypsin-EDTA) or using a cell scraper to detach the cells from the culture vessel. The detached cells are used for experiments or further seeding in different cell culture containers. Thus, these cells require periodic passaging	No need to use proteolytic enzymes or a cell scraper to detach cells, since these already prevail as a suspended culture. Once sufficient density of the cell suspension is achieved, the cell suspension would be divided into more new cell culture vessels with a fresh culture medium to increase the number of cells
Every cell culture container has a specific surface area and the growth of the cells is limited to that surface area only.	The growth of these types of cells is limited by the number or concentration of the suspended cells in the culture. So, an easy scale-up could be attempted.
Used for cytology, harvesting products continuously, and many research applications	Used for bulk protein production, batch harvesting in pharmaceutical/biotechnological industries, and many research applications

7.1 Medium Changes in Adherent Cells

In the case of adherent cells, the medium can be removed directly via aspiration through a vacuum pump aseptically fitted inside the laminar flow hood. Thereafter, the cell culture containers are refilled with fresh medium (Masters and Stacey 2007).

7.2 Medium Changes of Nonadherent Cells

Medium changes in nonadherent cultures involve **low-speed centrifugation** of the culture and **resuspending of the cells** in freshly prepared culture medium, generally in large volume containers (Masters and Stacey 2007).

8 Cryopreservation (Freezing) of Cultured Cells

Cryopreservation implies the **preservation of cells in liquid nitrogen** for an **infinite duration**, subject to the availability of a liquid nitrogen facility. Cell lines can be cryopreserved in a suspended state for indefinite periods provided a minimum temperature (**−135 °C)** is maintained (Baust et al. 2009).

The **main purpose** of cryopreservation is to keep stocks of cells with preventing the continuous need of maintaining cell lines in culture, at all times (Hay 1978;

Klebe and Mancuso 1983; Facklam and Geyer 1991; Wiebe and May 1990). It is especially beneficial when dealing with cells of a limited life span.

The other advantages of cryopreservation are as follows:

- Reduced costs (consumables and staff time).
- Reduced cross-contamination with other cell lines.
- Reduced genetic alteration and concurrent morphological changes.
- Feasibility of working with cells at a consistent passage number.
- Reduced microbial contamination.
- As and when necessary, thaw and freshly grow the cryo-preserved cells.

8.1 Procedures of Cryopreservation and Resuscitation

While cryopreservation of mammalian cells is a slow and step-by-step process, thawing or resuscitation is a rapid process. In the case of cryopreservation, the temperature is decreased at a rate of −1 °C to −3 °C/min, while in thawing, cells are directly put into a 37 °C water bath after taking out the cryo-vials from liquid nitrogen. They are kept in this state until the vial material thaws completely (generally within 2–3 min) (Mazur 1984; Baust 2002).

The following preconditioning is necessary for cryopreservation:

1. Change the medium before 24 h of freezing.
2. Use subconfluent cultures.
3. Use fresh healthy cultures with >90%viability and null microbial contamination.
4. Use at least 20% serum in the cell freezing medium.
5. Some freezing media could contain as high as up to 90% serum.
6. As a cryoprotectant of mammalian cells, 10% mammalian tissue culture grade DMSO can be used (Aswood-Smith and Friedmann 1979).

Although the precise requirement may vary with different cell lines, cell freezing or cryopreservation is a slow, step-by-step temperature-controlled process. Following a controlled rate of freezing (0 °C for 30 min, followed by −20 °C for 2 h, followed by −80 °C for overnight) in the presence of cryoprotectant like 10% DMSO where cells are preserved at −135 °C. Such ultralow temperatures can be obtained by specialized electric freezers or more usually by using liquid or vapor phase nitrogen (Coriell 1979; Farrant 1989).

NB: Liquid nitrogen and its vapor form is the best option for the preservation of mammalian cells. However, the major drawback of using liquid nitrogen is filling up the containers from time to time. Care should be taken not to accidentally spill over liquid nitrogen since it may burn the skin and also cause asphyxiation. Additionally, some reports claimed that during cryopreservation of cells in liquid nitrogen, viral contamination of the containers may happen.

Ultralow temperature storage is most commonly used ***in vapor phase nitrogen***. For vapor phase nitrogen storage, the cell vials are kept above in a box in a carefully

maintained shallow reservoir of liquid nitrogen (Smith 1981). A vertical temperature gradient is formed through the vapor phase, depending on the liquid nitrogen levels, the design of the vessel, and the frequency of container opening. In case the container is not maintained on regular basis, temperature variations in the upper regions of a vapor phase can be a significant issue. Modern designs of liquid nitrogen storage containers are better and have more consistent vapor storage capacity, ensuring thorough cryopreservation. Table 5 describes the salient features of various freezing methods/procedures.

The excess mammalian cells can be preserved in cryovial (**called cryopreservation**) in −80 °C or −150 °C deep freezers.

However, the best option for **permanent cryopreservation** is **vapor-phase liquid nitrogen**.

Cells are **gradually frozen** by first putting in a pre-chilled (**4 °C**) cell freezing medium for at least half an hour, then in −20 °C for at least 2 h followed by −70 °F to −80 °F storage overnight. Finally, the cells are permanently stored in liquid nitrogen. This is called **Cell Freezing** (Waymouth and Varnum 1976). In liquid nitrogen, cells retain their structure and function **up to eternity** as long as liquid nitrogen is regularly refilled.

8.2 The Cryopreservation of the Mammalian Cells Is Done Using the Following Steps

- **Step I:** Preparation of 100 ml mammalian cell preservation medium/cell freezing medium using the following materials:
  - **Cell culture medium:** 70 ml
  - **Serum:** 20 ml
  - **DMSO:** 10 ml

  NB: Medium must not contain any additives like antibiotics/antimycotics, growth factors, **etc.** ***DMSO must be of tissue culture grade with fewer***

Table 5 Ultralow temperature storage of cell lines

Method	Advantages	Disadvantages
Electric (−135 °C freezer)	Ease of maintenance Steady temperature Low running cost	• Requires liquid nitrogen backup • Mechanically complex • High storage temperatures relative to liquid nitrogen
Liquid phase nitrogen	Steady ultralow(−196 °C) temperature Simplicity and mechanical reliability	• Needs regular liquid nitrogen supply • High running costs • Cross-contamination risk by liquid nitrogen
Vapor phase nitrogen	No risk of cross-contamination by liquid nitrogen Low temperatures achieved Simplicity and reliability	• Needs regular liquid nitrogen supply • High running costs • Temperature fluctuations between −135 °C and −190 °C

impurities and moderate toxicity on human cells. One can replace 70 ml medium with serum, that is, 90 ml serum and 10 ml DMSO.

- **Step II:** Filter sterilizes the aliquot and stores the excess cryopreservation medium at −20 °C.
- **Step III:** Harvest the 70–80% confluent mid log cells between 4×10^5 and 8×10^5 cells/ml).
- **Step IV:** Count the cells using a **hemocytometer or automated cell counter** before resuspending in a fresh ice-cold cryopreservation medium at a final concentration of $2–5 \times 10^6$ cells/ml.
- **Step V:** Aliquot 1 ml cell suspension into each of either 1.5 or 2.0 ml cryovial.
- **Step VI:** Put the cryovial in −20 °C freezer for 2 h.
- **Step VII:** Following −20 °C incubation, the cryovial is kept in a −80 °C freezer overnight.
- **Step VIII:** Next day, the cryovials are transferred into liquid nitrogen, vapor phase, that is = −150 °C to −180 °C and liquid phase −196 °C.

The cells may be kept for years in a liquid nitrogen container.

NB: Nowadays, a programmable rate freezer is available.

Here are the steps for decreasing temperature in a programmable rate freezer:

- A programmable rate freezer can be started at 4 °C.
- Lower the temperature at a rate of −1 °C/min down to −30 °C.
- Set the rate to cool at −0.5 °C/min in between −30 °C and −50 °C.
- Set the rate to once again −1 °C/min between −50 °C and −100 °C.
- Once the cells are below −100 °C, they can be placed directly into liquid nitrogen storage.

8.3 Thawing and Revival of Cryopreserved Mammalian Cells

The t**hawing of cells** is done **very quickly** to expedite the thawing process. Cells are thawed rapidly in a 37 °C water bath. In this way, the ***ice crystals*** present in the **antifreeze** DMSO medium, melt very quickly so that they cannot damage cells, and cells are retained intact (Polge et al. 1949; Schroy and Todd 1976; Shannon and Macy 1973).

8.4 Thawing and Revival of the Cryopreserved Cells

- **Step I:** Set a water bath at 37 °C and allow the temperature to attain equilibrium.
- **Step II:** Take the cryovial from the liquid nitrogen and immediately put them into the 37 °C water bath. Thaw the cryovial as quickly as possible (within 1–2 min).
- **Step III:** Spray 70% alcohol on the vials and wipe out the alcohol using tissue paper.

- **Step IV:** Immediately take the vials in a laminar flow hood and add them to the complete cell culture medium.
- **Step V:** Make cell suspension by very slowly pipetting up and down without making any air bubbles.
- **Step VI:** Spread the cell suspension into a cell culture container and incubate at 37 °C, in a CO_2 incubator.
- **Step VII:** After O/N incubation, replace the cell culture medium containing DMSO with a fresh cell culture medium.
- **Step VIII:** Following step V, that is, after cell suspension preparation, it can be directly centrifuged (low-speed centrifugation) to remove the DMSO, thereby avoiding steps VI and VII.
- **Step IX:** Following centrifugation, add fresh cell culture medium, do a cell suspension, spread to a cell culture container, and incubate at 37 °C.

NB: Cells that are to be transported from liquid nitrogen over a long distance or time must be carried either in liquid nitrogen or dry ice. If transported in regular ice, the cryovial will slowly begin thawing of ice crystal, thereby harming the cells. This hinders the recovery of good viable cells. Although cell culture containers under culture can be checked at any phase using an inverted microscope, generally cell culture containers cannot be disturbed up to at least a couple of hours or overnight following the seeding of fresh cells in a new culture container. Too much handling or even mild shaking of cell culture containers may affect the attachment as well as conditioning of cultured cells.

9 Conclusions

This chapter describes the conceptual as well as practical understanding of primary, secondary, and continuous cell culture as well as adherent and nonadherent cell culture procedures. For primary culture, cells need to be isolated directly from mammalian tissues or organs before cell culture. When primary cultured cells are further subcultured into more culture vessels to get a greater number of cells, it is called a secondary cell culture procedure. However, every mammalian cell is capable of only a certain limited number of cell divisions after which the cells die naturally (**Hayflick effect**). That is why normal mammalian cells are said to be mortal. However, under laboratory experimental conditions such as using viruses, chemicals, or radiations, a normal mortal cell can be converted to an immortal cell by gaining the capacity of uninterrupted continuous division as long as nutrient supply and other culture conditions are maintained. Pathophysiologically, cells collected from tumor or cancer tissues have the capacity for uninterrupted or continuous cell divisions. Besides, most mammalian cells require attachment to the culture vessels before their growth and proliferation. However, few cells in the mammalian body such as B/T lymphocytes do not need any attachment for their growth and thus grow as a suspension culture. So, based on attachment or adherence, mammalian cells can be adherent and nonadherent. Understanding these cell culture

classifications is highly essential before learning about various mortal and immortal cells in the subsequent chapters. The chapter ends with a description of the freezing and thawing of cultured mammalian cells.

10 Cross-References

- ▶ Culture of Continuous Cell Lines
- ▶ Culture of Neuron and Glia Cells
- ▶ Isolation and Primary Culture of Various Mammalian Cells
- ▶ Primary Culture of Immunological Cells
- ▶ Stem Cell Culture and its Applications

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Isolation and Purification of Various Mammalian Cells: Single Cell Isolation

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Contents

Abstract

A typical adult mammalian body consists of 30–40 trillion cells. Mammalian cells differ in their shape, size, morphology, and other structural and functional characteristics. These characteristics of mammalian cells can be the basis for the isolation of a particular cell type from any tissue or organ. Except for blood

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_7-1

cells, various other mammalian cells are firmly adjoined together by extracellular matrix (ECM) proteins to form tissues and organs. To isolate and enrich the greater number of cells, their dislodging or enzymatic dissociation from the original parent tissues or organs is highly essential. Following dissociation, the isolation and purification of mammalian cells may be based on the following procedures: (1) Cell isolation is based on cell surface charge and adhesion. (2) Cell isolation based on cell size and density (density gradient centrifugation, filtration, sedimentation). (3) Cell isolation based on cell morphology and physiology (selective culture medium, laser capture microdissection system). (4) Cell isolation based on cell surface markers (fluorescence-activated cell sorting, magnetic separation, cell purification using complement depletion, purification methods involving DNA sequences, other technology: mitochondrial dye and aptamer technology). (5) Cell isolation based on a combination of the above techniques (immuno-density-assisted separation: erythrocyte Rosetting immuno-laser capture microdissection system, microfluidics-based cell isolation, hydrodynamic cell sorting, acoustic cell sorting, electrophoretic sorting of cells). ***In this chapter, besides describing all the above-mentioned isolation techniques, toward the end, various single-cell isolation techniques and the tools for single-cell analysis such as genomics, transcriptomics, and proteomics are discussed.***

Keywords

Single cell isolation · Flow cytometry/fluorescence-activated cell sorting (FACS) · Dynabeads based technology · Magnetic-activated cell sorting (MACS) · Laser capture microdissection (LCM) · Mitochondrial dye and aptamer technology · RBC rosetting · Immuno-laser capture microdissection system · Microfluidics · Hydrodynamic cell sorting · Electrophoretic cell sorting · Single cell genomics · Transcriptomics · Proteomics

1 Introduction

The basic concept of the structural formation of a mammalian body by cells, tissues, organs, and organ systems is already discussed in chapter ▶ "Mammalian Cell Culture: An Overview." A typical adult human body weighing about 70 kg may contain around **30-40 trillion cells**. In a human body, there are around **200 different cells** that are organized into several tissues and these tissues lead to the formation of various organs. The isolation and purification of a particular type of cells from **tissue or a solid organ** such as the liver require **digestion/disintegration** (**either by proteolytic enzymes or mechanical disintegration**) from the extracellular matrix (**ECM**), which is described in this chapter. **B**lood is a **liquid connective tissue** consisting of cells and plasma, without any solid ECM, and therefore blood cells can be directly isolated and purified without digestion/disintegration. Once various cells are separated from the ECM, the next step would be to isolate and purify the desired cells of interest.

Since mammalian cells vary in their ***shape, size, morphology, and other structural-functional characteristics***, these can be the b**asis for the isolation of a particular cell type** from any organ. Structurally, the mammalian cells have a plasma membrane consisting of **lipid-bi-layer** wherein the peripheral and integral proteins impregnated between layers and overall membranes **carry a net negative charge**, used for their isolation and purification. The various proteins in mammalian cell membranes are receptors, adhesion molecules, integrins, enzymes, porins, etc. with implicit biological functions. The utilization of **adhesion molecules and integrins** may be another mechanism to isolate mammalian cells. Besides, mammalian cells can also be separated through the **antibody produced against a specific cell surface protein**. Tyrosine/serine, threonine amino acids phosphorylation, is a characteristic event of eukaryotic proteins. Additionally, in the eukaryotic cells, some metabolic pathways are different from prokaryotic cells which can be helpful in the isolation and separation of mammalian cells.

This chapter describes the isolation of mammalian cells based on the following procedures: (1) Cell isolation based on surface charge and adhesion. (2) Cell isolation based on size and density (density gradient centrifugation, filtration, sedimentation). (3) Cell isolation based on cell morphology and physiology (selective culture medium, laser capture microdissection system). (4) Cell isolation based on surface markers (fluorescence-activated cell sorting, magnetic separation, cell purification using complement depletion, purification methods involving DNA sequences, other technology: mitochondrial dye and aptamer technology). (5) Cell isolation based on a combination of the above techniques (immuno-density-assisted separation: erythrocyte resetting immuno-laser capture microdissection system, microfluidics-based cell isolation, hydrodynamic, acoustic, and electrophoretic sorting mechanisms).

The following factors affect the selection of a particular experimental method for cell isolation: (1) Vulnerable/sensitive properties of the cells, (2) Comparative stress magnitude (mechanical/chemical/physiological) which the chosen cell type can withstand without any undue effect on its viability, (3) The extent of cell purity and culture yield required, (4) Bearable contamination threat (practically null, if the associated cells are needed for culture), (5) Negative or positive isolation methods, (6) Implicit needs of the downstream applications, such as cell culture, nucleic acid/protein extraction, etc., (7) Expenses incurred and management of labor, reagents, instrumentation, etc.

Toward the completion of this chapter, we discuss the various single-cell isolation techniques. Two major needs fulfilled by isolated single cells in the present scientific era are (i) screening of unusual cells in a heterogeneous population and (ii) genomic, transcriptomic and proteomic characterization, at the cellular level (Cell: the fundamental structural and functional unit). Besides the single-cell isolation mechanisms, this chapter also discusses the tools necessary for single-cell analysis (Recktenwald and Radbruch 1998; Amos et al. 2011; Tomlinson et al. 2013; Almeida et al. 2014).

This chapter will enhance the reader's knowledge regarding the isolation and purification mechanisms of mammalian cells, including single-cell isolation.

2 Collection of Tissues/Organs from Animal or Human Subjects

The first step of mammalian cell culture is to collect suitable tissue/organ for isolation and purification of cells.

Tissues/organs can be collected from the following sources:

- Euthanized experimental animals.
- Human organs discarded (disease organ removed by surgery, e.g., removal of breast cancer tissue from breast cancer patients).
- Human organ replaced for transplantation (e.g., nonfunctional lung/heart/kidney, etc.)
- Umbilical cord discarded at the time of baby birth.
- Human organs are ethically collected from accidentally dead persons.
- Human aborted fetuses are ethically collected with proper permission.

Note the Followings:

- Before the collection of human tissues/organs, the research protocol must be approved by the ethical committee apart from fulfilling the consenting norms from the donor.
- The human organs must be freshly collected (***within 2–3 h of death after removal from a mammalian body***), otherwise cultured cells generally lose the capacity to grow.
- The ethical committee must approve the type and number of animals being used for the experiment.
- Before the collection of any organs from any animal, **euthanasia** is essential. Euthanasia (Greek, meaning ***"Good Death"***) is performed to induce painless death. However, these procedures are quick, easy to perform, and do not cause histological or histochemical changes in the cells that may affect scientific results.
- The reagents and equipment used for euthanasia must be safe (nontoxic) and inexpensive.

Common techniques for euthanasia are as follows:

Carbon Dioxide Asphyxiation
Pentobarbital Overdose
Exsanguinations (to Drain of Blood)
Cervical Dislocation

While **Carbon Dioxide Asphyxiation** is a painless technique but it may cause an effect on the tissues. **Cervical Dislocation and Exsanguinations** have little or no effect on the animal tissues/cells and are, therefore, preferred.

3 Dissociation/Disintegration of Tissues/Organs into Cells

Two processes for isolation of cells from mammalian organs/tissues are as follows:

Enzymatic Dissociation
Mechanical **Disintegration**

Here is a brief discussion of the above techniques:

3.1 Enzymatic Dissociation

- Organs are made up of various tissues and tissues are made up of various cells.
- Following tissues/organ collection, they are subject to treatment with various proteolytic enzymes (e.g., **trypsin/collagenase, etc.**).
- Enzyme-based disaggregation allows hydrolysis of fibrous connective tissue and the ECM.
- Currently, the enzymatic method is extensively used as it offers high recovery without affecting cell viability.

The following paragraphs briefly describe the controlled proteolytic digestion of tissues/organs for dissociating or disintegrating mammalian cells:

Trypsin-Based Disaggregation or Trypsinization
Either crude or pure trypsin can be used for proteolytic digestion. The treatment procedure can be attuned to either warm trypsinization or cold trypsinization.

Warm Trypsinization
- Take a fresh piece of tissue/organ.
- Wash with PBS.
- Add trypsin (concentration and volume may vary from cell to cell type. Some examples are presented in chapters ▶ "Isolation and Primary Culture of Various Mammalian Cells," ▶ "Primary Culture of Immunological Cells," and ▶ "Culture of Neuron and Glia Cells").
- Incubate at 37 °C for 30 min with very mild stirring.
- Collect the supernatant, centrifuge, and suspend in medium and culture.

Cold Trypsinization
- Take a fresh piece of tissue/organ.
- Wash with PBS.
- Add with cold trypsin for 6–24 h.
- Remove the trypsin.
- Put the tissue at 37 °C for 30 min.
- Collect the residual trypsin with dissociated cells.
- If necessary repeat the 37 °C incubation.

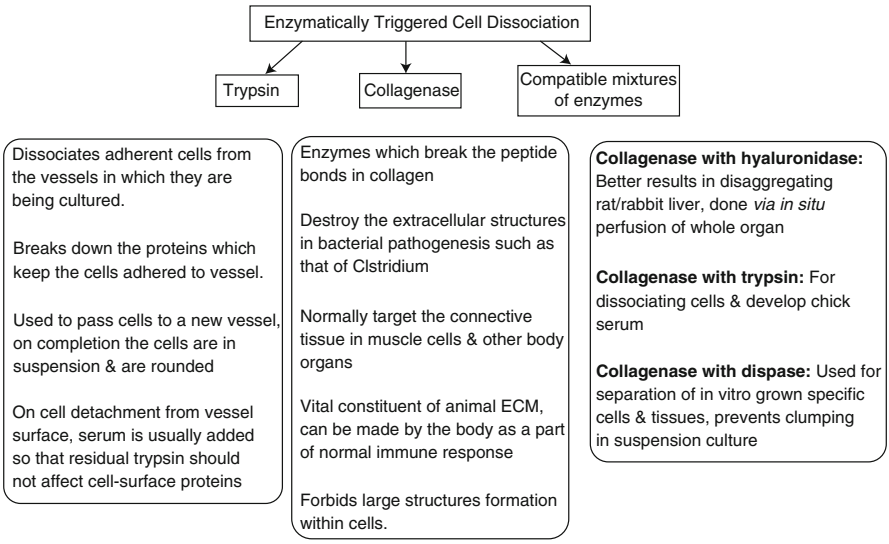

Fig. 1 Summary of various enzymatically triggered cell dissociation mechanisms

NB: Cold trypsinization-dependent cell damage has fewer chances of getting more viable cells in this process than warm trypsinization.

Limitations of Trypsin Disaggregation

Trypsinization can unduly harm certain cells, like epithelial cells. On many occasions, it is not favorable for certain tissues (e.g., fibrous connective tissue). Nevertheless, this constraint can be easily resolved by choosing some other enzymes for cell lysis.

Collagenase-Based Disaggregation

- Take a fresh piece of tissue/organ.
- Wash with PBS.
- Add crude **collagenase** (an enzyme for triple helix collagen breakdown).
- Combined use of **collagenase** and **hyaluronidase** generally suits better for disaggregation of rat or rabbit liver, which could be accomplished through in situ perfusions of the entire organ.
- Many investigators have also used **trypsin** and **collagenase** in combination, for dissociating the cells.
- Some researchers use **collagenase and dispase** combinations for specific tissues.
- To date, collagenase-driven cell disaggregation has been attempted on multiple human tumors, such as tumors that originated in the brain, lungs, and other mammalian organs.
- The concentration and time of incubation with collagenase vary from tissue type.
- Figure 1 summarizes the various enzymes and their combinations used for cell dissociations.

3.1.1 Erstwhile Options

Besides the above-mentioned enzymes, a range of other suited options including bacterial proteases (such as **dispase, pronase**, etc.) have been screened. However, the results have not met the expectations of a good outcome. Subsequently, though, **hyaluronidase** and **neuraminidase** have garnered the interest of late and promise well compatibility for use in conjugation with the other enzymes.

Note the Following:

- Controlled proteolytic treatment disaggregated the adherent cells from the collected organs and tissues.
- The cell isolation protocol may vary with the E**C**M composition, cell structure, and type being contained in different tissues.
- The isolation procedures and protocols for various cells (e.g., primary, immunological, or continuous) are described in chapters ▶ "Isolation and Primary Culture of Various Mammalian Cells," ▶ "Primary Culture of Immunological Cells," ▶ "Culture of Neuron and Glia Cells," ▶ "Culture of Continuous Cell Lines," and ▶ "Stem Cell Culture and Its Applications" with details of proteolytic digestion.

3.2 Mechanical Disaggregation

- Generally, loosely associated cells in the organs like the spleen, liver, etc. can be easily isolated via mechanical disaggregation.
- It is necessary to disaggregate soft tissues such as soft tumors. The mechanical approach involves slicing or harvesting tissue and subsequent harvesting of spilled-out cells.
- This can be achieved via **sieving, syringing, and pipetting**.
- This procedure is inexpensive, rapid, and simple. However, all the above-mentioned approaches involve the risk of cell damage and thus mechanical disaggregation is only used when the cell viability in the final yield is not very important.

4 Methods of Isolation and Purification of Various Mammalian Cells

The isolation of a particular cell type from a heterogeneous population depends on the unique properties of that cell type.

Here five major cell isolation techniques are discussed

1. Cell isolation based on surface charge and adhesion.
2. Cell isolation is based on size and density.
3. Cell isolation is based on morphology and physiology.

4. Cell isolation based on surface markers.
5. Cell isolation is based on a combination of the above techniques.

Here are the details of the above techniques:

4.1 Cell Isolation Based on Surface Charge and Adhesion

- A detailed discussion of adherent and suspension cells is made in the previous chapter, that is, in chapter ▶ "Mammalian Cell Culture Types and Guidelines of Their Maintenance." The techniques used for adhesion-based cell separation can be **fast and inexpensive**.
- Briefly, in general, most mammalian cells maintain a negative charge on their surface. However, the charge distribution may vary from cell to cell type. The presence of **phosphatidyl serine** and **Donnan membrane equilibrium** (unequal distribution of ions across the biological membrane) may be the reasons suggested for the negative charge of the plasma membrane. Negative charge confers adhesive properties to most cells, in the mammalian culture grade **polystyrene-**made culture vessels (Petri plate/flasks, etc.).
- As discussed in chapter ▶ "Mammalian Cell Culture Types and Guidelines of Their Maintenance," while most mammalian cells (such as endothelial cells) are **strictly adherent** in nature, some like MCF-7 breast cancer cells are **loosely adherent** and even a few (e.g., B-lymphocytes/T-lymphocytes) are **non-adherent** and grow in suspension.
- ***The adherent cells perish without the support of an attachment surface.***
- The adherent feature determines the extent of cell attachment to plastic and other polymer surfaces that can be used to separate adherent and suspension/free-floating cells.
- Isolation of both adherent and free-floating cells can be tailored to the specific cell type by adding the requisite growth factors and suitable cell culture plates.
- In certain applications, such as studies involving circulating cells or unpurified mesenchymal stem-cell samples, it could be useful to use adhesion as a selection criterion for sub-population sorting.
- Monocytes are quite useful for regenerating post-infarction myocardium, owing to which, using **microprinted** or **adsorbed adhesion** or **matrix proteins** could be crucial for screening the finest populations.
- A substantial proportion of present-day progenitor research using mesenchymal stem cells distinguishes the adherent and non-adherent cell populations (Didar and Tabrizian 2010).

4.2 Cell Isolation Based on Size and Density

Three physical methods for isolation and separation of mammalian cells are based on their size and density.

They are as follows:

- Density gradient centrifugation
- Filtration
- Sedimentation

Familiar procedures in this domain include density gradient centrifugation and filtration. These are sometimes also referred to as **"bulk sorting,"** due to the capability of these methods to isolate comparatively large cell populations in a reasonable time. The high-yield provision of the bulk sorting method sometimes is used as an essential component of multiple clinical and biological protocols, where a large number of sorted cells are generally not needed. However, it must be noted that the purity and homogeneity of harvested cells are considerably lower than those using other cell separation protocols. For example, *Miller and associates* demonstrated the velocity sedimentation-driven cell separation in 1969 (Miller and Phillips 1969).

Here is a brief discussion of the above three methods:

4.2.1 Density Gradient Centrifugation

- Density gradient centrifugation (**DGC**) is a frequent procedure used as an initial step to enrich many cell populations. For subsequent separation and harvesting, refined methods like **FACS, MACS**, or single-cell sorting could be used.
- Fundamentally, cell sorting could be accomplished based on cell sizes and density differences in a defined centrifugation culture medium.
- Separating the mixed cell population based on implicit cell sizes and nature could be accomplished via a layered assortment of medium layers, with successively decreasing densities within a given sample. This is facilitated using low-speed centrifugation.
- Adult progenitor cells originating from bone marrow are usually intensified in this way only.
- A similar procedure can be extended to augment cardiomyocytes from embryonic stem cell-derived populations even though the ultimate purity is not adequate.

The basic steps of density gradient centrifugation are as follows:

- Take the suitable sample mixture of cells.
- Mix with a suitable gradient medium.
- Centrifuge in a **swinging bucket rotor** with intended speed and time.
- Cells would be fractionated and separated based on their densities.
- **Heavier cells** usually pellet down while the **lightest ones**, along with the **dead cells, float** on the gradient top.

Working Theory of Density Gradient Centrifugation

- The sedimentation frequency of cells in a suspension varies directly with the applied centrifugal force (g) and inversely with the medium viscosity, η.

However, in the case of unchanged g and η, the rate of sedimentation varies with the characteristic cell size and the differences between medium and cell densities.

- The general relation between sedimentation rate, g, and η, is explained by the following equation:

$$V = \frac{d^2\left(\rho_p - \rho_o\right)}{18\eta} \times g;$$

where v = sedimentation rate, d = cell diameter, g = centrifugal force (acceleration due to gravity), ρ_p = cell density, ρ_o = medium density, and η = medium viscosity.

- The typical medium for cell separation in course of DGC consists of the gradients including the densities of all possible cell-type mixtures.
- In course of the centrifugal process, each cell kind sediments to the identical density domain of the gradient termed an isopycnic point.
- In accord with the above equation, when the densities of the medium gradient phase equal that of cell type, the typical sedimentation rate becomes zero, resulting in the settling of cells at the instantaneous phase rather than at the tube bottom.
- Therefore, cells are separated only based on density, no matter what the sizes be.

The gradient used in density gradient centrifugation is of two types:

Continuous gradient
Discontinuous gradient

Here is a brief description of the two types of gradients:

Continuous Gradient

- Continuous gradients are described by a periodic change in density from the top to the bottom of the tube. These can be supposed as consisting of an infinite number of interfaces. Thereby, isopycnic cell band formation transpires at a precise cell density.
- The creation of a continuous gradient is often tedious, necessitating the need for high- and low-density solutions which could form a linear gradient on being mixed. This gradient spans the range between the limits of starting solutions.
- More importantly, a continuous gradient enables a greater resolution owing to the increased chances of isopycnic cell banding caused by the wide ranges of available densities. Furthermore, the separation of cells having closely matching densities mandates the need for a narrow gradient range.
- Some cell types isolated by this process are **Leydig cells, intestinal epithelial cells,** and **BM cells.**

Discontinuous Gradient

- A discontinuous gradient comprises distinct bands with implicit densities, increasing steadily from top to bottom. Discontinuous gradients, on the other hand, manifest significant flexibility and simplicity of usage.

- Usually, a cushion of Percoll or a single step is needed to accomplish splendid augmentation of a specified target cell.
- The separation medium is usually diluted to a range of varying densities before being layered in decreasing order from bottom to top.
- This kind of density gradient exhibits sharp interfaces between the bands and is usually considered when a sharp cell band is needed at a particular interface.
- Blood cells represent the best example of cells separated by a discontinuous gradient (Ulmer and Flad 1979).

Frequently used gradient media and their specific applications:

The following attributes must be possessed by an ideal gradient medium:

- Adequate density range for isopycnic banding of cell types.
- Ionic strength, pH, and osmolarity should be physiological.
- Viscosity should be low.
- There should be Nontoxicity.
- Inability to invade biological membranes.
- Capability to give rise to continuous and discontinuous gradients.
- Easier removal from isolated cells.
- No critical effect on downstream assay or culture procedures.

NB: The density gradient method is robust, logical in operational procedure, and not much expensive. However, on being used independently, it results in below-par cell purification and therefore is rendered infeasible for similar-sized implicit cell populations.

4.2.2 Filtration

- In 1964, ***Seal S. H.*** used the filtration technique for the first time to separate large tumor cells from the smaller blood cells (Seal 1964).
- Filters are available in various pore sizes.
- Filters having specific pore sizes are used. The cells smaller than the pore size pass through the filter, while the larger ones are retained on the filter.
- So, filtration-based cell separation is a size-driven method. There are a few commercially available filtration devices that have specifically been developed for cytological purposes.

Cell mixture can be passed through the filter based on either of the following force types:

Passive capillary action.
Gravitational forces.
Active motorized vacuum pump (Table 1).

Benefits of Filtration Separation

- The assay should be robust, easy to follow, and reproduce.
- Cater to high throughput and yield.
- Filters possessing captured cells can be directly used in downstream assays.

Table 1 The different commercial-grade cell filtration units

Device	Pore size (μM)	Sample volume (mL)	Principle
ISET	08	10	Vacuum pump
Metacell	08	50	Capillary action
Screencell Cyto	7.5	3	Vacuum pump
Screencell MB	6.5	3	Vacuum pump
Screencell CC	6.5	6	Vacuum pump

Inadequacies of Filtration Separation

- The assay should have poor specificity and purity.
- Uncommon cells are frequently lost with a common outcome of false positives.
- The surface phenotype of certain cancer cells is lost during the process, complicating the downstream assays.

Applications of Filtration-Based Cell Separation

Cancer cells are usually large in size and irregular in shape. So, filtration is the best method to isolate the cancer cells, in particular for ***circulating tumor cells (CTCs)***.

To study the various CTCs, filtration can be simultaneously combined with the following assays:

- **Immunostaining**
  This technique can be used on the filter trapped cells directly for screening the suspected CTCs via specific biomarkers. The method employs a pedigree of implicit probes to distinguish the epithelial, mesenchymal, or other cells, based on **CTCs origin**.
- **The screen cell systems**
  This system can be configured with culture vessels and a microscope to spontaneously culture the trapped tumor cells in filters, keeping them under observation.
- **Fluorescence in situ hybridization analysis**
  Fluorescence in situ hybridization **(*FISH*)** analysis can be made promptly on the filter to assess chromosomal rearrangements of the cells.
- **The screen cell BM system**
  This configuration has inbuilt collection tubes to make way for prompt DNA and RNA analysis of trapped CTCs.
- **Microfiltration method**
  Microfiltration is an entire passive sorting method using cell size and its ability to pass or get obstructed across the micropores. Cells smaller than the selected pore size are filtered across and are collected in a micro-well rather than the larger ones which are trapped in the "sieve." Microfiltration devices are designed to sort out the diverse cell populations at once using micropore arrays of varied target sizes. Furthermore, the precision of this procedure can be enhanced by labeling the cell populations with density bead-tagged specific antibodies.

4.3 Cell Isolation Based on Morphology and Physiology

Every cell type has its implicit morphology and physiology.
Cells can be separated based on the following features:

Shape and morphology
Specific histological staining
Selective culture/growth medium
Selective physiological response

NB: The discussion of cell characterization based on shape, morphology, and culture medium design is illustrated in the primary culture of mammalian cells (chapter ► "Isolation and Primary Culture of Various Mammalian Cells") and culture of immortal cells (chapter ► "Culture of Neuron and Glia Cells").

4.3.1 Selective Culture/Growth Medium

A specific cell type can be selected over the undesired cell populations via culturing in a specific medium that provides a selective advantage to the desired cell type. In mammalian cell culture, we can selectively encourage the growth of specific cell types by the addition of the following entities:

Specific growth factors
Specific cytokines
Addition of antibiotics
Inclusion or exclusion of specific metabolites (for auxotrophic cell culture)
Addition of specific metabolism and growth inhibitor (e.g., aminopterin for hybridoma cell culture)

Here, briefly, the usefulness of selective growth medium and antibiotic resistance in the selective cell culture is discussed.

Theory of Selective Growth

- Analysis of gene function often mandates the creation of mammalian cell lines which **over-express either a transgene** or a **mutated gene**.
- **Similarly knockout** of both gene copies abolished its prevalence or its product (**protein**) from the system.
- A **marker gene** is generally inserted into a cloning vector and the chimeric vector before being transfected into a specific cell type. These transfected cells are thereafter cultured in a selective culture medium.
- So, the cells which merely express the selectable markers possess a growth benefit and can easily gain the dominant clone type, within a few weeks of culturing.
- There are two approaches to using a selective growth medium based on selectable markers.

They are as follows:

Antibiotic resistance and
Metabolic/biosynthetic enzymes

Here is a brief discussion about them:

Antibiotic Resistance

If the transfected cells possess an antibiotic resistance gene, the specific antibiotic is added to the medium at a concentration that is lethal to the cells which do not carry the resistance gene. Antibiotic resistance gene-containing cells will survive, but not the cells that do not contain any antibiotic resistance gene. Frequently used antibiotics for selecting mammalian cells include **bleomycin, puromycin,** and **hygromycin**.

Metabolic/Biosynthetic Enzymes

This strategy is best illustrated by the hypoxanthine-aminopterin-thymidine (**HAT**) selection medium, normally used in hybridoma technology for monoclonal antibody (**mAb**) generation. Here aminopterin blocks the de novo DNA synthesis, whereas hypoxanthine and thymidine provide raw materials for the alternative "**salvage pathway**."

The chemical structures of aminopterin, hypoxanthine, and thymidine are shown in Fig. 2, which are hydrophilic precursors for the formation of DNA nucleotides. Hypoxanthine Guanine Phosphoribosyl-Transferase **(HGPRT**) is the prominent requirement for the salvage pathway and the cells which implicitly express the HGPRT gene only can survive in the HAT medium (Table 2).

Benefits of Selective Medium Isolation

Growth selection of specific clones is a customary task in most cell and molecular biology labs, offering the following advantages:

- Performance regulation and simplicity.
- Reproducibility.
- Adequate cell yield.

Concerns Behind Selective Medium Isolation

- The threat of contamination.
- The emergence of spontaneous resistant clones not carrying the gene of interest.
- Expenditure on procuring some reagents and specialized medium.
- Time and skilled manpower are needed for the growth and development of relevant clones.

4.3.2 Laser Capture Microdissection System

Laser capture microdissection (**LCM**), also known as **microdissection**, **laser microdissection** (**LMD**), or **laser-assisted microdissection** (**LAM**), is a technique for isolating explicit cells from microscopic locations of tissues/cells/organisms

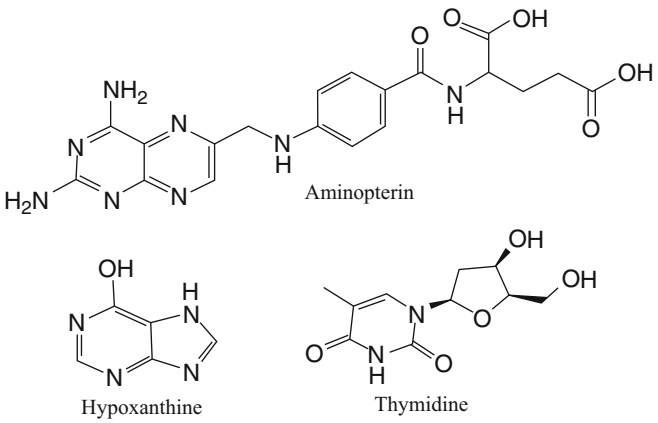

Fig. 2 Chemical structures of aminopterin, hypoxanthine, and thymidine, depicting a hydrophilic sensitivity

Table 2 Common selective markers of mammalian cell culture along with the selection criterion

Target cells expressed markers	Selection conditions	Mechanism of selection
Adenosine deaminase **(ADA)**	9-β-D-xylofuranosyl adenine (Xyl-A)	Xyl-A can be converted to Xyl-ATP. This leads to the death of cells. Cells bearing ADA can detoxify Xyl-A to its inosine derivative
Aminoglycoside phosphotransferase **(APH)**	G418, an aminoglycoside antibiotic	G418 is fatal to cells due to its protein synthesis blockage while APH hinders G418 activity
Cytosine deaminase **(CDA)**	5-fluorocytosine (5-FU)	CDA converts 5-fluorocytosine to 5-fluorouracil, inhibiting the proliferation
Dihydrofolate reductase **(DHFR)**	Methotrexate (MTX)	MTX is a potent competitive inhibitor of DHFR. It is essential for purine synthesis. Using a high concentration of MTX selects the cells expressing high DHFR
Histidinol dehydrogenase **(hisD)**	Histidinol instead of histidine	Histidinol does not support cell growth and is toxic. The hisD converts histidinol to histidine
Thymidine kinase **(TK)**	HAT (hypoxanthine, aminopterin, thymidine)	Aminopterin blocks nucleotide synthesis. TK expression is needed to use the salvage synthesis pathways via hypoxanthine and thymidine
Xanthine-guanine phospho ribosyltransferase **(XGPRT)**	Xanthine, hypoxanthine, aminopterin, thymidine	Same as for TK

(**dissection on a microscopic scale using a laser**). This method for isolating a pure sample from a heterogeneous mixture enables more efficient and accurate outcomes, enabling multiple downstream microeconomics utilities such as **next-generation sequencing, Sanger sequencing, PCR, and proteomics**.

This technique was described for the first time, in the early twentieth century. The method involves slicing the cells of interest from tissue sections, using a thin laser beam. A slightly more refined microscopic configuration, termed Laser Capture Microdissection (**LCM**), was subsequently expanded in the National Institutes of Health (**NIH**), Bethesda, Maryland, USA, by ***Liotta and Emmert-Buck*** for isolating pure cell populations from heterogeneous tissues. This isolation is primed based on **natural morphology** or the **implicit histological/immunological staining**.

In this assay, optical manipulation and cell categorization involve striking a focused laser beam whereby the cells can be trapped due to the distinct refractive indices of a cell and its surrounding fluid. This difference paves way for optical scattering, forcing the cells away from the light source. At the same instant, the radiation pressure gradient draws the cells to the highest intensity or, more simply, the focusing maxima.

As and when the gradient overcomes light scattering, the cells move close to the maxima, getting trapped in the "optical tweezers." Optical sorting principles are practiced to adapt FACS on a miniaturized scale. Microfluidic optical tweezers are also used for accurate and precise single-cell isolation (refer to the last section of this chapter). Optically induced cell sorting micro-configurations are also in practice which use more than a single laser light wavelength to configure the multiple cell populations. In general, cell separation using optical tweezers is driven via size, fluorescent intensity (native or from labeled antibodies), and the strength of a laser.

LCM equips the researchers to accurately and precisely isolate the tumors from healthy tissues, stem cells from the stroma, and epithelial cells from the parenchyma. Associated with high-precision surgery methods, LCM can even be used for single-cell separation. Recently, several state-of-the-art advances have been accomplished in LCM devices for numerous diverse applications such as genomics, proteomics, tissue regeneration, cancer cell characterization, diagnostic pathology, and fundamental cell biology.

The utilities demanding primary cell isolation rely on fluorescence-assisted laser microdissection as a significant negative selection method. Procedurally, laser dissection employs computer software to guide a medical laser for the ablation of non-fluorescent cells in a heterogeneous composition of cells. While explanting tissues from transgenic animals possessing fluorescent reporter gene(s), laser dissection can be used to isolate the corresponding cells.

The non-fluorescent cells of specific requirements are isolated following the labeling with fluorescent antibodies or via fluorescent dye microinjection or reporter plasmids, before being selectively isolated from contaminated cells. The laser used for ablation could be used to accomplish microinjection.

Utilizing the LCM cell purities to the extents of $>99\%$ could provide 90% cell yields from a 10^1 to 10^8 ranged sample sizes through in situ laser purification. More fundamentally, laser microdissection is also suited to preserve the neuron viability in

course of purification rather than conventional techniques such as FACS, an emerging threat prone to fragile cell type. Still, the FACS are useful and helpful for isolating the primary cells which are intended to be used as reagents. A potential concern while using laser technology is its expensive infrastructure and rather highly specific applications for usage with a requirement of exposing and labeling the cells with fluorescent markers (Emmert-Buck et al. 1996; Schutze and Lahr 1998; Fend et al. 1999; Espina et al. 2006, 2007; Berns 2007; Nakamura et al. 2007).

Types of Laser Capture Microdissection Systems

The LCM system which can be either manually or robotically manipulated has the following sub-units:

An inverted microscope
A laser diode
A laser control unit
A joystick-controlled stage
A CCD camera and
A computer

LCM systems are of the following two types: **INFRARED LCM (IR-LCM)** and **ULTRAVIOLET LCM (UV-LCM).** A combined automated IR/UV system is also available by some companies, such as **Arcturus Veritas Instruments**. The minimum laser beam diameter of an LCM microscope is 7.5 μm while the maximum diameter is 30 μm. The tissues can be maximally heated to 90 °C and also for a few milliseconds, thereby leaving the cellular structure and macromolecules intact. Here is a brief discussion of the LCM types.

Infrared Laser Capture Microdissection

This LCM configuration was first developed by Emmert-Buck and colleagues, at the NIH, and was launched as the PixCell module by Arcturus Engineering. The IR-LCM design comprises a thin transparent thermoplastic ethylene-vinyl acetate (EVA) film over a sliced tissue section. After the visual screening of the tissue region of interest, the cells are conjoined with the film using short IR laser pulses. Practical action is workable through lower cell-slide adhesion compared with cell film, enabling the specific cells to be plucked off. Eliminated cells are thereafter shifted to a microcentrifuge tube having apt buffer solutions for downstream assays (Emmert-Buck et al. 1996).

Ultraviolet Laser Capture Microdissection

The only difference between this LCM configuration (more famous as UV-LCM) is the incidence of a narrow UV laser beam (instead of IR) onto the sample-specific region after visual screening and the ablation of unwanted cells in the vicinity of desired cells. The invention of this mode is credited to *Schmitz and Lahr* in 1998 before commercialization by the PALM Zeiss Microlaser Technologies. The operation commences with the tissue mounting on a 6 μm thick membrane before being

placed on a microscopic glass slide. Thereafter, the desired cells are pulled up and harvested in an overhanging cap. As the UV-LCM configuration does not involve any non-specific adherence of desired cells, this mode is preferred over the IR variation, for some applications (Schutze and Lahr 1998).

Sample Preparations for Laser Capture Microdissection

The samples used for LCM are as follows:

Blood smears
Live cells from cell cultures
Fresh tissues
Frozen tissues
Formalin-fixed paraffin-embedded tissues
Metaphase spreads

NB: Frozen or paraffin-embedded tissue sections are the most frequently used samples for optimal capture and screening.

- ***While cutting the sections using the cryostat/microtome, the optimum temperature should be maintained.***
- ***The tissue sections should be ideally within 5–15 μm in thickness.***
- ***Sections thinner than 5 μm would be too fragile, while*** **those** ***thicker than 15 μm would not be dissected properly.***
- Dissected tissue sections should be subjected to a standardized staining procedure followed by a sufficient extent of dehydration so that they are distinctly visualized and amicably stored.
- In general, the **formalin tissue** is comparatively more suited than its formalin-fixed counterpart, as it safeguards the RNA, DNA, and proteins sans any sort of cross-linkage attenuations.
- In most of the pathology labs across the globe, formalin-fixed tissue sections are designated as standards for LCM-based cancer cell biomarker investigations.

Benefits and Cautions of Laser Capture Microdissection

Benefits of Laser Capture Microdissection

- The most obvious positives of LCM methodology include its high throughput, accuracy, and compatibility with diversified tissue sources and their associated uses.
- The variations in laser potential difference and tissue morphology could facilitate a collection of thousands of cells in a relatively limited period.
- Accurately directed laser beams minimize the damage to adjacent tissues, allowing a single tissue section to be probed as many times as possible.
- LCM microscope and variations (based on light sources) are generally simple to operate and exhibit an easy-to synergize ability with other assays.

- Several distinct tissue types can be analyzed using LCM via distinct provisions such as regular hematoxylin and eosin-stained slides and archival sections besides the immunologically stained frozen/fresh tissues.
- Used lasers are in general lowly powered, posing no significant risk to macromolecule structure and integrity.
- LCM harvested cells can be efficiently used for downstream assays, which need active nucleic acids and proteins (Koller et al. 2004).

Cautions of Laser Capture Microdissection

- For eliminating the chosen cells after laser targeting, the tissue sections cannot be cover-slipped. Without mounting media and coverslips, the refractive index of targeted tissue could be unduly affected. This attenuation renders it cumbersome to observe the targeted cells. This bottleneck aggravates tissues having amorphous morphology such as those belonging to tumors and lymph. In these tissues, there are no architectural markers owing to which capturing the specific cell type is rendered tedious. Nevertheless, improved staining modules like that of immune-staining could be optimized to moderate such trivial hurdles.
- Most of the LCM modules incorporate the lasers wherein minimal spot size is 7.5 μm, typically not adequately precise to isolate the single cells. Still, new and better platforms are being screened and evaluated for making single-cell LCM from tissues, albeit for cytological preparations, regular lasers could also efficiently dissect the single cells.
- Yet another formidable constraint of tissue drying (specifically for frozen tissues) is the insufficient adherence of dissected tissue onto the film or inverted cap. For IR-LCM configuration, a low laser power might not be potent enough to melt the membrane over the tissue.

Specific Uses of Laser Capture Microdissection

- Emerging utility and scientific interest in LCM over the past few years have been the reasons for keenly pursued genomic, transcriptomic, and proteomic analysis of several different cells from diverse tissue samples.

Ahead are its distinguished features for its significance in cancer research:

- Malignant tissues are greatly heterogeneous concerning cell composition.
- Cancer originating cells and cancer stem cells are precariously weakly characterized, needing still better identification signatures.
- Neoplastic alteration manifests chronological mutations and clonal progression.

Specific Utilities of Laser Capture Microdissection

Cancer Diagnostics

The LCM modules are swift provisions to isolate cells from tumor tissues before their use for molecular characterization and tumor genotyping. The techniques have been efficiently used for screening gene alterations, deletions, and loss of heterozygosity in multiple tumors like prostate, melanomas, lymphomas, and others.

Assessment of Chemotherapeutic Treatment of Cancer

LCM exhibits suitability for screening randomly chosen tumor cells apart from biomarker analysis so that the unrelenting clones escaping chemotherapy could be identified. Manifold assay platforms of the LCM, with whole-genome microarrays and RT-PCR conjugation, have distinctly screened the novel biomarkers for invasive **glioblastoma, breast cancer, ovarian cancer,** and others. Apart from this, CM has also been in use for developmental biology, embryology, and xenografting technology.

4.4 Surface Markers Enabled Cell Isolation

Usually, the least invasive method is chosen for isolating and sorting cells in experimental biology or bioengineering studies. On many occasions, such procedures use the cells' surface proteins, via their expression and functionality distinctions. This is accomplished to purify a sub-population from heterogeneous cell mixtures using ***FACS, magnetic sorting, or adhesion-driven cell separation***.

Alternately, surface protein-driven cell purification could be used as a mechanism for negative selection like that of complement exhaustion to retrieve a population not having any undesired cell types. Surface protein-instigated sorting is a significant tool for multiple research projects owing to its least risk to cell phenotype and viability although it does have some implicit limitations.

In the following paragraphs, we step by step describe the usefulness of fluorescence-activated cell sorting (**FACS**) in the isolation and separation of cells using cell surface marker proteins. Besides FACS, other cell separation techniques described in this section are magnetic separation, cell purification by complement depletion, purification methods involving DNA sequences, mitochondrial dye, and aptamer technology.

The techniques described here are as follows:

Fluorescence-activated cell sorting (**FACS**)
Magnetically activated cell sorting (**MACS**)
Cell purification by complement depletion
Purification methods involving DNA sequences
Other technology: mitochondrial dye and aptamer technology

Here is a brief discussion about the above techniques:

4.4.1 Fluorescence-Activated Cell Sorting

- Fluorescence-activated cell sorting (**FACS**) is a method of separating cells by selectively tagging them with colored fluorescent dyes bound to specific cellular structures or molecules. It provides a method for **sorting** a heterogeneous mixture of biological **cells** into two or more containers, one **cell** at a time, based upon the specific light scattering and **fluorescent** characteristics.

- The technique of FACS was invented by *Borner and Herzenberg* for isolating viable cells using fluorescent probes. The first commercial instrumentation therein was named Becton Dickinson Immunocytometry Systems during the 1970s. Over the years, such automation has witnessed significant up-gradation to the scale of considerably refined high-throughput systems which could quantify as many as 12 fluorescent colors at the same instant of time. The populations thereby stained using distinct fluorophore-conjugated antibodies can be segregated using discrete fluorescent signals generated corresponding to wavelengths and intensities. FACS is extensively used for positive selection-driven cell isolation (Bonner et al. 1972).
- The technique uses phenotype-based implicit surface markers along with fluorescent antibodies. Cells warranting purification are thereby subjected to a fluorescent excitation laser before being separated via one or multiple fluoresced cell surface proteins.
- FACS performs a decisive role in multiple purification methods requiring intracellular fluorescent marker induction like that of green fluorescent protein (GFP), bypassing the need for protein-specific antibodies.
- A complement functional analog fluorescence-activated cell sorting (FACS) of FACS equipment involves the use of forwarding and side scattering optical systems to distinguish the cells vis-à-vis sizes and internal complexity. Of note, such functions are much less specific compared to antibody-driven FACS and exhibit significant utility as provisions of separating the different cells as per their viability.
- The expenditure associated with FAC equipment, in general, forbids each laboratory from procuring its individual FACS instrumentation. Still, the conventional uses of the method compensate for the inter-departmental FACS core working mode at most institutions.

  NB: The operational procedure of FACS allows reasonably high throughput, disappointingly, the sorting sub-unit of the probe eventually becomes unrealistic with small sample sizes.
- The working accuracy of FACS instrumentation implicitly depends on the sample to sheath pressure stoichiometry as well as the number of markers distinguishing the various populations. In general, a higher sample to sheath pressure ratio sorts out large cell numbers in relatively less time albeit the accuracy of cell sorting and cell viability are trivially reduced. This is due to rather infrequent habituation, whereby two cells move across the detectors at the same fluorescence-activated cell sorting (FACS) instant. Likewise, overall sorting accuracy is lowered with each subsequent marker to describe a cell population (Orfao and Ruiz-Arguelles 1996; Herzenberg et al. 2002; Vahey and Voldman 2008; Will and Steidl 2010; Wang et al. 2011).

General Cell-Labeling Protocol

- A single-cell suspension is prepared in FACS buffer (ice-cold phosphate buffer saline or PBS), 10% FCS, and 1% sodium azide at 10^6/100 μl cell number.

NB: Do not add sodium azide to buffers if there is a concern regarding recovering cell functions, that is, if the cells are to be collected for functional assays. Such a practice inhibits metabolic activity; to prevent this, 0.5–1% BSA can also be added to the buffer.

- For fluorescence-based staining, transfer the cells in 12 × 75 mm^2 **polystyrene round-bottomed Falcon tubes**.
- Other containers such as test tubes, Eppendorf tubes, and 96-well round-bottomed microtiter plates could also be used but round-bottomed Falcon tubes exhibit a wide preference.
- Supplement each tube with 100 μl cell suspension.
- At this stage, obstruction using antibodies could be attempted but ideally should be considered if cells exhibit high extents of F_C receptors, resulting in non-specific binding and background fluorescence.
- Include 100 μl F_C block (diluted in FACS buffer at 1:50 extent) to each sample.
- Ice-incubate all cells for 20 min.
- Centrifuge at 1500 rpm for 5 min at 4 °C.
- Get rid of the supernatant.
- The cells are incubated with the labeled antibody (0.1–10 μg/ml) cocktail over the ice, keeping away from direct light for an optimum incubation period.
- If at all essential, dilutions should be made in 3% BSA/PBS carrying FACS buffer (propidium iodide can be added at this step for getting rid of dead cells).
- Incubate for a minimum of 30 min in a dark environment, either at room temperature or 4 °C. This step necessitates standardization.
- Cells are subjected to triplicate washing via 5 min of centrifugation at 1500 rpm.
- Resuspend the cell pellet in 200 μl to 1 ml ice-cold FACS buffer.
- The cells are maintained in dark, either on ice or at 4 °C (within a refrigerator), for the designated analysis duration.

Following is the modified procedure, in case a primary unlabeled antibody is being used:

- Dilute the fluorochrome-conjugated secondary antibody using FACS buffer as per the manufacturer's instructions). Resuspend the cells in this solution followed by their incubation for a minimum of 20–30 min, either at room temperature or 4 °C in the dark. Wash the cells thrice via 1500 rpm centrifugation for 5 min before their re-suspension in 200 μl to 1 ml ice-cold FACS buffer. Thereafter, the cells are kept either on ice or at 4 °C (within a refrigerator) for the designated analysis duration.
- If at all, there is a need of preserving the cells for some days or the analysis reveals human or bacterial contaminations, avoid re-suspending the cells in 200 μl to 1 ml ice-cold FACS buffer. Rather, add 100 μl (1–4%) paraformaldehyde before subjecting to 10–15 min of incubation, at room temperature.
- Centrifuge the samples at 1500 rpm for 5 min before their re-suspension in 200 μl to 1 ml ice-cold PBS.

- Fixation inevitably inactivates most biohazardous agents, reduces the risk of deterioration, and assures maintenance of cell integrity. The relative quantity of fixative needed for various samples needs optimization by the user.

Analysis

To minimize the errors and arbitrations, the cells are screened using flow cytometry at the earliest possible before being analyzed at the earliest (we suggest the very same day) (Fig. 3). For storage till 16 h and increasing flexibility in the cytometry planning time, the cells are re-suspended in 1–4% paraformaldehyde to minimize the deterioration (Table 3).

- A fluorophore typically works via light absorption at a particular wavelength following which its electrons are excited to a higher energy state. Due to the transient stability of the higher energy state, the probe undergoes a conformational change amidst which excited electrons are back triggered to the initial low-energy state. The difference between the energy of these states (higher–lower) is the energy emitted as fluorescence or emission.
- The corresponding wavelength deviation of the higher and lower energy states is also termed "Strokes Shift."
- The chronological events of excitation and emission occur multiple times, with the output being read as a fluorescent signal.

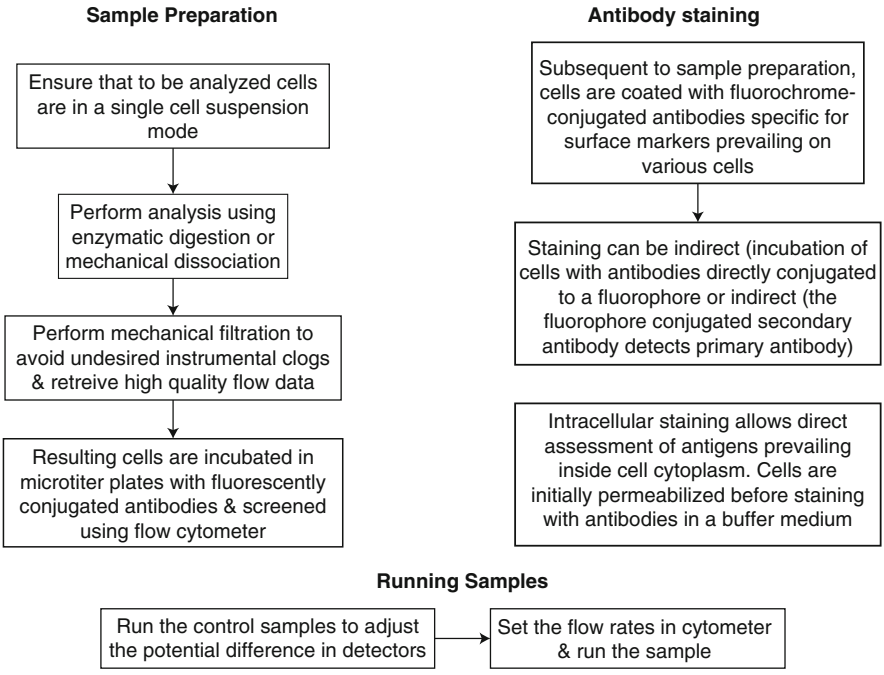

Fig. 3 The different steps in a flow cytometry operation. Antibody staining is performed after the initial sample preparation

- Because emitted light has low energy than the excited one, the emission wavelength of a particular fluorophore is longer than the excitation wavelength, resulting in an altogether distinct color.
- It is essential to energize the fluorophores at their respective excitation to raise their emission intensity.

The Working Principle of the Flow Cytometer

FACS instrumentation can be divided into two parts, namely, **fluidics** and **optics**. While fluidics allows the cells to flow in a stream, optics detects them.

Here is a very brief discussion of FACS fluidics and optics:

Fluorescence-Activated Cell Sorting Fluidics

- The objective of a fluidic system is to configure a cell suspension as a uniform single-cell stream that could discretely detect the cells and be sorted afterward.
- The fluidic system typically consists of a central core across which the sheath fluid flows under high-pressure conditions before finally enfolding the sample fluid.
- As the sheath fluid is subjected to high pressure, it moves a bit faster than the sample, resulting in drag over the sample fluid.
- This hydrodynamic force on the sample generates a single stream, allowing the implicit screening of cells as and when they move across the illumination source.

Fluorescence-Activated Cell Sorting Optics: Lasers and Filters

Table 3 Excitation and emission wavelengths of various FACS fluorophores

Compound	Excitation (nm)	Emission (nm)
DAPI	358	461
GFP	395	508
CFP	458	480
FITC	494	518
7-AAD	546	647
APC	633	660
APC-Cy7	633	780
Rhodamine 123	507	529
PE-Cy7	480, 743	767
PE	480, 565	575
Hoechst 33342 and 33258	352	461
Pacific Blue	410	455
PerCP	490	675
PerCP-Cy5.5	490, 675	695
	507	529

AAD 7-aminoactinomycin D, *APC* allophycocyanin, *APC-Cy7* APC-Cyanine7, *CFP* Cyan Fluorescent Protein, *DAPI* 4', 6-diamidino-2-phenylindole, *FITC* fluorescein isothiocyanate, *GFP* green fluorescent protein, *PE* phycoerythrin, *PE-Cy7* phycoerythrin-cyanine 7, *PerCP* peridinin-chlorophyll-protein, *PerCP-Cy5.5* peridinin-chlorophyll-protein-cyanine 5.5

- After the formation of every single stream, each cell moves through distinct laser beams and is sorted as per its scattering and fluorescence emission characteristics.
- Each laser generates a single light wavelength at a specific frequency, based on which the lasers could be classified as ***ultraviolet, violet, blue, yellow, green, red, and infrared***.
- On being excited by a laser beam, a fluorophore emits a signal at varying wavelengths which subsequently passes through designated optical filters.
- Every filter, in reciprocation, permits only certain specific wavelengths to move across, resulting in their capture by photo-diode detectors and subsequent quantification.
- The majority of modern-day flow cytometers are composed of three to five lasers along with multiple optical filters per laser. Such a configuration facilitates the recognition of multiple signals at the same time instant.
- Besides the fluorescence, scattered light is also analyzed concerning cell size and granularity. Distinct wavelengths thereby generate photon signals for capturing by the photo-multipliers (PMTs). These signals are converted into proportional electronic pulses, recorded as "events."
- These pulses are digitized via an electronic processing system before being stored as FACS files. A typical pulse is quantified all through its height and area, inferring the corresponding signal intensity and width, symbolizing a cell's spent duration for which a cell is an incident with a laser beam.

Electrostatic Sorting of Fluorescently Labeled Cells

Besides fluidics and signal detectors, FACS instruments are additionally equipped with the following accessories:

A. The provision to generate cell droplets (often via high-frequency vibrations).
B. Charged plates for deflecting the generated cell droplets onto the specific collection tubes.

Five Essential Steps of Cell Sorting

- The cells move across the laser beams to generate the implicit fluorescent signals, which are subsequently detected as illustrated in the previous section.
- The cell containing the stream is fragmented into droplets, each of which captures a cell.
- Droplets move within the deflection plates.
- Charged droplets carrying the cells (as embedded) are deflected in the electrostatic field before being harvested.
- Uncharged droplets are retrieved via collection in a waste chamber.

Advantages of Fluorescence-Activated Cell Sorting

- At the present juncture, FACS is the most accepted and standardized cell purification method. Its wider trust is attributed to its significant efficiency and accuracy.

- The most anticipated use of FACS is for separating the cell populations that could be used as experimental models in both basic sciences and translational studies.
- Cell sorting based on FACS is presently a standardized procedure in numerous research and clinical laboratories.
- As the instrumental and software analysis specifications mount, larger applications revert to FACS for the isolation of multiple cells.
- FACS is a very sensitive and high-throughput method for cell isolation from heterogeneous populations.
- This is an ideal facility for sorting multiple cell populations at the same instant, based on their immuno-phenotype.
- This technique encompasses a flexible methodology that sorts the cells not only based on surface markers but also based on cell size and granularity, cell cycle status, intracellular cytokine pattern, metabolic status, etc.

Limitations of Fluorescence-Activated Cell Sorting

- Though FACS aids in sorting out multiple cell populations at the same instant, the sorting process in itself is a slow event. This is because shear forces are disadvantageous making the cells detrimental. So, it is desired to moderate the stream flow rate for ensuring cell viability and an adequate sorting speed.
- Revival of the majority of FACS sorters is ~50–70%, mandating the commencement of sorting with a high cell number. This conditioning manifests a challenge for the sorting of stem cells and circulating tumor cells (normally, the rare cells).
- Mounting complexity and precision extents of FACS reciprocate in high chances of technical inaccuracies. The infrastructure and handling expenses of FACS sorters are high and its instrumentation necessitates the involvement of highly skilled manpower for training, troubleshooting, and repair.
- As the FACS mandates single-cell isolation, it provides no information regarding tissue architecture and intracellular interactions.
- Although advanced FACS sorters enable a simultaneous screening of **10–12 colors**, it mounts the bottleneck of fluorophore "spillover" across the non-specific channels. This is caused due to the coinciding fluorophore spectra, lowering the resolution between the closely related immuno-phenotype populations.

Major Drawbacks of Fluorescence-Activated Cell Sorting

- The application of FACS is restricted to surface marker protein detection. In particular, for the needs of **developmental biology** and **tissue engineering**, several proteins that are critical to distinguishing the cell populations are intracellular. Thereby, these can't be easily labeled with antibodies in live cells.
- Some cells may be a little excessively sensitive to mechanical stimulation, resulting in their perishing amidst FACS sorting.
- Quite often, it is very difficult to screen and analyze the surface proteins expressed on characteristic cells associated with a definitive phenotype or development stage.

NB: Surface protein-based FACS is useful only if one can enumerate the differential cell surface marker expressions.

Specific Applications of Fluorescence-Activated Cell Sorting

- In the last few decades, development and awareness of FACS functioning has significantly steadied the **hematopoiesis, stem cell biology, and oncology** advances.
- Besides, the high-speed sorters have simplified the retrieval of certain specific clinical blood cell populations.
- FACS is a highly important requirement of various blood cell research efforts. A great deal of knowledge regarding the biological understanding of various hematopoietic cells, adult stem cells, and progenitors. The hierarchical morphology of the hematopoietic system is derived from the highly specific cell sorting and configuring procedures (Fig. 4).
- Isolation of various cancer cells, like those of cancer stem cells (CSCs), metastatic cells, circulating tumor cells (CTCs), and blasts, using characteristic immunological phenotypes is substantially done via FACS.
- Enhancement of transfected cells, a familiar task in many biomedical laboratories, acutely depends on FACS.
- High–throughput speed sorters are frequently used by clinicians for separating blood cell fractions such as lineage negative or CD^{34+} populations for ex vivo management and transplantation.

4.4.2 Magnetically Activated Cell Sorting

- Immuno-magnetically induced cell separation relies on the deflection of cells under the influence of a magnetic gradient. Of note, magnetic responsiveness of

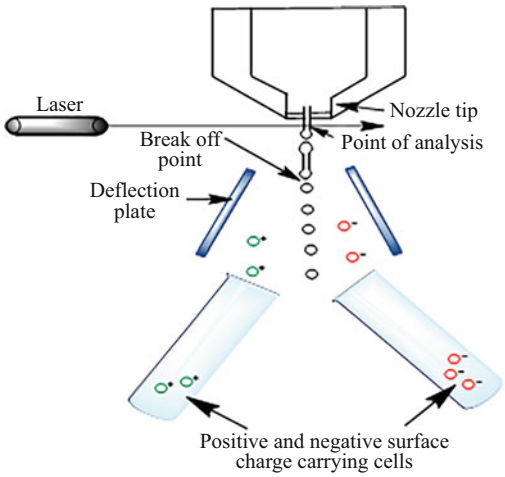

Fig. 4 Description of different FACS components used in cell sorting

cells could be intrinsic (like the iron comprising RBCs) or due to antibody-coated superparamagnetic particles targeted against specified antigens.
- Immuno-magnetic protocols can be used for both positive and negative kinds of cell selection.
- In magnetically induced cell sorting, the cell labeling could be direct (using primary antibodies coated beads) or indirect (using non-conjugated primary antibodies and conjugated secondary antibodies) (Abts et al. 1989; Miltenyi et al. 1990; Handgretinger et al. 1998; Carroll and Al-Rubeai 2005; Grützkau and Radbruch 2010).

Based on the type of magnetic particles used, immuno-magnetic separation is mainly of two kinds, as follows:

Dynabeads-based technology.
Magnetically activated cell sorting technology.

Here is a brief description of them:

Dynabeads-Based Technology
- **Dynabeads** are typically hollow, spherical superparamagnetic polymeric particles having a homogenous size distribution with a functionally suited surface for conjugating the multiple bioactive molecules or cells.
- These were coined and unveiled in the quest to create uniformly sized polystyrene spherical beads by *John Ugelstad*, in 1976 at the University of Trondheim, Norway.
- The credit for understanding and wider implementation of Dynabeads working principle goes to Dyno Industry in 1980, after which this technique has been used for isolation and engineering of biomaterials such as cells, nucleic acids, proteins, and pathogenic microbes.
- Salient features of these structures include the uniformity in sizes, shapes, and surface area for a reproducible performance and the least chemical agglutination.
- Dynabeads are quite familiar probes for cell isolation. The important cell types of interest which are purified using Dynabeads include leukocytes (**CD^{4+} T cells**), stem cells, or CTCs.
- These designer probes could be covalently conjugated with an antibody that binds with a specific protein on the targeted cell surface.
- The Dynabeads could also be attached indirectly to the cells, either via conjugated streptavidin linkage to a biotinylated primary antibody or a secondary antibody that is linked with the primary antibody.
- Streptavidin linkage to a primary antibody facilitates the Dynabeads for cell capture with lower surface protein activity (Ugelstad and Hansen 1976).

Cell Separation Properties of Dynabeads

- Dynabeads could assist in cell separation in conical tubes, micro-tubes, and, perhaps, even in multi-well plates. The multi-well plate configuration is well compatible with being fitted into the customized magnets.
- Working size of **Dynabeads** ranges from 1 to 3 μm.
- These beads display a magnetic sensitivity only if kept inside a magnetic field with no residual magnetism. That is, these lose magnetism once the applied magnetic field is removed.
- Non-specific interactions (including the chemical agglutination) with this probe are minimized by the defined surface chemistry.
- The polymeric shell in these beads guards the target cell against Fe exposure.
- Biological retrievers (including cells, proteins, and nucleic acids) could be separated by conjugating or adsorbing bioactive molecules to the bead surface.
- One illustration for such an application is the use of Thermo-Fisher 11445D with CD4 antibody for extracting out the mouse $CD4^{+}$ T cells. The same principle could be extended to several other ligands for tracking or controlling specific cells via immunoprecipitation reactions.
- Similarly, the cells could be incubated with an amicable antibody-coated beads cocktail inside a magnetic field to isolate the bound and unbound cells. For unbound target cells (negative selection), the aspiration works out, while for positive isolation, bead-conjugated target cells are washed before being eluted out (Neurauter et al. 2007).

4.4.3 Magnetically Activated Cell Sorting

- Magnetically activated cell sorting (**MACS**) is a procedure for separating the different cell populations based on their surface antigens. This method was invented by **Miltenyi Biotec**, wherein MACS is the registered trademark.
- Since the MACS development in the 1990s, the technology slowly emerged as a standard paradigm in cell culture laboratories for robust cell isolation.
- The isolation of targeted or non-targeted cells is governed by the selection approach, which is labeled with 50 nm magnetic microbead conjugated antibodies, which are henceforth put under the influence of a magnetic field.
- *Boettcher and associates* for the first time separated hematopoietic stem and progenitor cells from the entire mouse bone marrow, bearing anti-CD^{117} magnetic beads from Miltenyi.
- Likewise, *Chopra and colleagues* positively selected the CD^{14+} cells using Miltenyi (130-050-201) from human blood/buffy coats and thereafter separated neutrophils via negative selection with Miltenyi (130-097-658) from mouse bone marrow.
- In another suited illustration, Garrett Bakelman and associates used Miltenyi (130-097-048, 130-097-057, 130-097-055) to retrieve **CD^{4+}, $CD8^{+}$, and CD^{19+} cells**, respectively, from the twin astronauts of NASA (Garrett Bakelman et al. 2019).

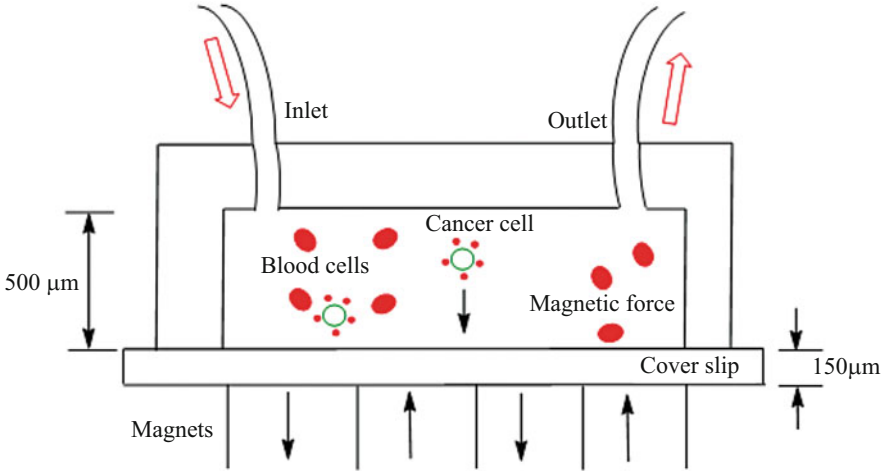

Fig. 5 Magnetically triggered separation of cancer cells from blood cells

- Figure 5 (above) depicts a magnetically controlled separation of cancer cells from a mixture of blood cells.

The three platforms available for providing the magnetic gradient are as follows:

- Magnetic separators
- MACS columns
- AUTOMACS

Here is a very brief discussion about them:

Magnetic Separators

Separation using magnets is made using the shapes wherein tubes of suitable sizes could be fitted. This methodology comprises the simplest MACS approach in which incubation is succeeded by keeping the tubes inside the magnet for a designated duration. Subsequently, the unbound fraction of cells is exhausted and the bound cells persist, leaving the target counterparts in the bound/unbound state.

NB: The working principle of magnetically triggered cell sorting relies on antibody specificity which separates the sub-populations of cells from complex composition mixtures. The characteristic feature involves the association of antibodies with surface proteins* via *magnetic conjugates (and not the conventional fluorophores).

- Thus, the cost-effective and easy-to-engineer equipment involving an electromagnet or permanent magnet could be used to isolate the cells bearing the surface marker of interest. This setup makes the cost of setup half extent of that of FACS equipment.

- Although conventional magnetic sorting does not manifest any phenotypic changes, it is still feasible to energize the surface protein-driven phenotypic variations deliberately by subjecting the surface proteins to mechanical forces through bound magnetic beads.
- The major concerns of surface protein-driven purification method are endured to a greater extent in course of magnetically aided sorting, as very fewer number of antibodies remain available for sorting the cells compared to FACS with the sorting provision of one marker at a given time instant.
- Furthermore, studies also reckon a low accuracy of magnetically driven cell sorting contrary to that of FACS in the context of false positives (substantially attributed to cell–cell adhesion) and false negatives.
- Due to the greater use of magnetically aided sorting for bulk enrichment which is not similarly precise as that for FACS, the improvement of magnetically aided sorting to retrieve highly pure cell populations (matching the clinical needs) is quite unlikely.

MACS Columns

- These columns comprise a ferromagnetic sphere matrix for a considerable enhancement of the magnetic field, while the mixed-cell population is placed within a magnetic separator.
- These columns work via injecting the cells into columns so that they can move freely inside the inter-spherical regions.
- The labeled cell population could be isolated in the suspension region (magnetically unbound), whereas unlabeled cells pass through and could be effortlessly retrieved.
- On removing the column from the separator, bound cells could also be eluted out.

Columns enable the following major benefits compared to the conventional separators vis-à-vis positive selection:

A. Increment in the magnetic strength modulates the detection sensitivity, particularly for minimally labeled cells, and
B. Cells are under much low stress as these do not bind magnetic spheres while being in labeled configuration.

AUTOMACS

The AUTOMACS is developed by Milteni Biotech. This provision is an automated version of MACS column separation assembly and could be engineered well for multiple sample sorting.

Benefits and Cautions of Magnetic Separation Benefits

- MACS is a high-throughput, specific, and robust provision for separating target cells or getting rid of unwanted cells.
- The columns in MACS aid in the isolation of rare cells with high specificity.

- The method can be scaled up or down, as per the yield desired and the downstream processing requirements.
- The method could be diversified along with the multiple platforms, including that of microfluidic devices.

Cautions

- Sorting using Dynabeads mandates the elution of beads as their large sizes could unduly affect the downstream operations.
- Separation of cells using magnets is not very accurate and efficient as it often leaves a certain extent of unbound cells in the tube along with the bound cells.
- To obtain a high purity in the output sample, the sorting step needs multiple repetitions.
- Interactions with magnetic particles may induce shear, causing a loss of morphology in delicate cells.

  NB: To minimize the undue shear effects, MACS columns should be used rather than the separators.

Explicit Utilities of Magnetically Activated Cell Sorting

- Automotive MACS is a familiar operation at the clinic level for obtaining specific blood cell populations in bulk. This method provides a higher cell yield than FACS.
- Magnetically induced cell separation is the preferred module to get rid of undesired cells from mixed-cell populations before trying the more sophisticated methods of FACS, which have higher accuracy.
- Not merely restricted to getting rid of undesired cells, MACS could also be used to intensify some cell populations like that of T cells, monocytes, and others, before commencing the in vitro culture.
- For instance, Greenwood and associates attempted the isolation of human monocyte-derived primary macrophages. The investigators firstly obtained white blood cells using Ficoll-Paque centrifugation. Henceforth, isolated cells were subjected to positive selection using anti-CD14 beads (130-050-201) and LS columns (130-042-401) from Miltenyi.

Cell Purification Using Complement Exhaustion

- Complement diminution represents an erstwhile method to accomplish cell purification, utilizing the monoclonal antibodies to get rid of undesired cells in a mixed population.
- Very briefly, the method uses activated plasma proteins to generate pores in targeted cell regions using proteolytic surge. These cells are eventually lysed and washed away from the sample.
- The typical procedure is 95–100% accurate in eliminating the specified cell populations.
- The method is not preferred by the majority of researchers having access to FACS equipment, as FACS provides a more reliable mechanism to accomplish a selected cell population.

- Anyhow, the approach still offers plentiful distinctions of interest for cell purification.

DNA Sequences Mediated Cell Purification Techniques

- Despite the proven significance of surface proteins in distinguishing one cell type from another, constraints of specificity, availability, and limiting cross-reactivity badly hurdle antibody technology.
- To overcome such bottlenecks of antibodies, several alternative strategies have come to the fore.
- Two commonly employed gene modulation techniques for cell purification comprise the activation **of sortable marker expressions, in turn manifesting opposition to selection agents**.
- A major benefit of these techniques is their feasibility of working with positive and negative selection, which significantly improves the efficacy of cell isolation.
- In this method, the transgenic expression could also be accomplished through minimal promoter and the precise condition-based enhancer sequence.

Four main strategies have been developed to address this question:

- Homologous recombination
- Zinc finger recombination
- Viral infection and labeling
- Integrase and transposons

Here is a very brief discussion about the above strategies:

Homologous Recombination

- This terminology gained eminence in context with the creation of transgenic animal models. The typical methodology involves the generation of a DNA segment having a reporter and DNA sequences similar (or homologous) to them being targeted gene. This segment is subjected to electrolysis and ultimately administered to embryonic stem (ES) cells. Gradually, as the cells divide, the homologous regions break and result in translocation followed by integration within the genome. The amicably labeled cells are selected via a drug-resistant response manifested by the introduced DNA sequence.
- Amidst the process of creating transgenic animals, the ES cells are injected into allogeneic blastocysts, generating the chimeric animals. These chimeric animals are thereafter back crossed to generate animals having a stable reporter expression. The manner of this recombination generally uses reporter plasmids having certain homologous regions corresponding to a particular gene. The typical expression of some specific genes can thereafter be quantified all across an animal via screening the reporter signal expressed in the place of the targeted gene.
- Compared to the viral vectors, the homologous recombination technique enables a site-specific integration of reporter sequences rather than the incorporation within the random gene sequences. In general, the reporters introduced via

homologous recombination are accurately expressed concerning the target gene. No transgenic leftover prevails from the administered reporter cassette. Homologous recombination restricts the size of inserted sequences since the larger plasmid is prone to degrade before getting stably incorporated within the genome.

- Disappointingly, the efficacy of integration (for human cells) in course of homologous recombination is abysmally low. Animal models created via this procedure for the expression of reporter proteins could be used for isolating specific cell populations.
- An alternative approach to homologous recombination is the use of bacterial artificial chromosomes (BACs), typically allowing the use of larger sequences. This technique renders the insertion of large reporter sequences feasible concurrently along with the apt upstream and downstream promoter and enhancer sequences. Thereby, using BACs enables the substitution of a reporter gene into the specific position of the gene of interest, for improved insertion of genetic material besides an enhanced integration efficacy.
- A series of alternative protocols are proposed to automate and expedite the use of homologous and BAC recombination(s). Such advantages not only enhance their acceptability in experimental biology and translational science laboratories but also pave way for their rapid commercialization.
- A further provision to enhance the working accuracy of homologous recombination is the use of zinc finger nucleases via considerable enhancement of select chromosomal double-strand breaks, leading to a significant enhancement in the foundational events of homologous recombination. This is made possible by designing the plasmids encoding zinc finger nucleases to induce breaks at specific loci within the double-stranded DNA.
- Thereby, double-strand breaks can be introduced within a target cell in the vicinity of a specific gene, enabling the insertion of large reporter constructs via homologous recombination at significantly high efficiency with no serious concerns equivalent to the viral approaches. A potential concern of homologous recombination is the difficulty to fabricate the custom site-specific zinc finger endonucleases. In the absence of well-designed endonucleases, there could be a non-specific induction of double-strand DNA breaks, leading to apoptosis.

Non-integrating Vectors

Transitory transfection with pluripotency encoding constructs (genes) has been used to induce pluripotency in target cells although the technique could be well suited for reporter constructs for cell purification. In one approach, cells are subjected to electrophoresis and thereafter rendered to plasmid vectors which gain an entry within the cells and ultimately express the encoded gene after their degradation. Similarly**, episomes** are constructed to encapsulate fabricated plasmid vectors, enhancing vector stability. This, in turn, improves the opportunity for cellular access to the vector and the successful delivery of its payload. Each method currently suffers a low efficiency which often results in complicating the incorporation of large reporter sequences under the control of cells like specific promoters.

Viral Infection and Labeling

Over the past several years, viruses are being increasingly preferred for their ability not only to carry but also to transfer the gene to host cells. A significantly large number of viruses capable of delivering reporter sequence to a cell population could be generated via incorporation of the desired genetic reporter sequence along with essential viral sequences into specific host cells (e.g., HEK 293T cells). Thereafter, cells expressing the reporter sequence could be isolated from the larger heterogeneous population using selection methods like FACS or enrichment via drug resistance. Three viruses are at present routinely used for an everlasting integration of reporter gene sequence into other genomes, namely:

- Retrovirus
- Adenovirus
- Lentivirus

Here is a brief discussion about them:

Retrovirus

Using RNA as its genetic material, this virus infects a host cell via enzyme reverse transcriptase (RT) which converts its RNA into a complementary single-stranded DNA (cDNA), which is again converted to dsDNA. Subsequently, the retrovirus incorporates its DNA into the genetic material (DNA) of the invaded host cell, paving way for its replication. Human immunodeficiency virus (HIV) is presently the most well-studied and known retrovirus. A virus like this specifically targets the dividing cells and rather poses difficulty in infecting non-dividing or comparatively weakly dividing cells, such as the neurons.

Adenovirus

These are the medium-sized, non-enveloped viruses having an icosahedral nucleocapsid enclosing a double-stranded DNA genome. The nomenclature of this virus originates from their inceptive isolation from human adenoids in 1953. ***Human mastadenovirus C*** **is** the best-known adenovirus. These viruses are significantly smaller than several other of their counterparts, carrying sequences that are merely 4.8 kb in length. Perhaps, many reporter sequences possessed by these are too long for being delivered by the **adeno-associated viruses**. The inherent genome compatibility of adenoviruses forbids the delivery of large sequences such as those needed for reporter constructs. Recent attempts of making high-capacity adenoviruses draw an interest herein, making adenoviral technology an interesting arena for cell purification. The benefit of not causing permanent integration (of their genetic material) into the invaded host genome makes the adenoviruses suitable for time-bound activation of explicit tissue gene expression, including those in neurons.

Lentivirus

- Lentiviruses are the specific retroviruses (having RNA as genetic material) that are relatively recent in terms of their development, having the ability to infect the dividing and non-dividing cells equally well. This characteristic feature of lentiviruses (which use integrase to transduce the host cells). These vectors necessarily require integration into the host genome for the expression of the vector. Oligonucleotides till 8 kB genome can be packed within a lentiviral vector, possessing significantly high transduction ability with mutagenic ability.
- These viruses exhibit a high efficacy in infecting the dividing and non-dividing cells, having a relatively large payload. The integration caused by these viruses in the host genome is highly random.
- The lasting integration of genetic markers within a host can be highly useful in experimental biology although it is a negative force for some therapeutic applications. As a result, this ability restricts the Lentivirus's utility in generating purified cell populations. Of late, certain configurations have come to the fore allowing selective removal of lasting integrated sequences using Cre-loxP, as an aftermath of phenotypic sorting. Thereby, despite the excision of reporter sequences, small transgenic sequences persist within the target genome, manifesting the risk of tumor formation.

Integrase and Transposons

Solutions to address the size limitation issue of certain vectors are enacted via the use of plasmids encoding **integrases** and **transposases**. The underlying principle of these remedies involves a transient expression of an enzyme that is capable of inserting a synthesized reporter sequence in the host genome at the particular insertion loci.

The phiC31 Integrase

The phiC31 integrase control is noticed in bacteria, making it feasible to exert a reasonable control on the reporter sequence expression. This enzyme works through certain sequences within a mammalian genome, which are similar to bacterial attP integration sites. At these locations, the phiC31 integrase controls the integration of sequences containing an attB site at the attP loci within the bacteria, together enabling the insertion of **attB-containing reporter sequences**. The number of such insertion sites in a mammalian genome is low and these prevail primarily within the introns (not contributing to transcription). As a result, reporter sequence insertion using this technique is quite unlikely to disrupt the endogenous gene transcription.

NB: Activity of phiC31 integrase is irreversible; thereby reporter sequence insertion using this approach is permanent and likely to pose restrictions in clinical studies.

The Transposon

The transposon system uses a large synthesized reporter sequence **characterized by the** inverted transposon recognition sites along with a non-integrating plasmid encoding transposase. This transposase controls the random insertion of a reporter sequence in a host genome, enabling the reporter expression tuned cell sorting. On the completion of cell sorting, an erstwhile transient transfection via transposase encoding vector removes the inserted reporter sequence. Some prominent transposon systems used in mammalian models are Tol2, Sleeping Beauty, Frog Prince, and piggy BAC. While transposon systems like piggy BAC can be eliminated from a genome without any residual transgene persistence (in the targeted cell), the reversible functioning of transposases mandates a further sorting step. In this step, cells that are to be used in humans are essentially separated based on successful reporter construct removal.

Other Purification Methods: Mitochondrial Dye and Aptamer Technology

Several useful techniques are known for being acquainted with large quantities of cellular reagents, as is the case for tissue engineering and cell therapy. Using **mitochondrial dye** and **aptamer technology** are the frontrunners herein.

Mitochondrial Dye

- Using mitochondrial labeling ***Hattori and colleagues*** described the specific labeling of cardiomyocytes derived from pluripotent stem cells. In the study, tetramethyl-rhodamine methyl (**TMRM**) ester (a Nernstian dye), capable of mitochondrial entry and exit by mitochondrial membrane potential, was used by *Hattori and colleagues* as a positive selection marker for cardiomyocytes derived from **rat, mouse, and human pluripotent stem cells**.
- Of note, cardiomyocytes represent the cells, prevailing in the majority within the heart. The availability of primary cardiomyocytes for research purposes remains acute, in light of which, the mitochondrial dye remains a robust, non-genetic mechanism to generate a significant cardiomyocytes population with $>99\%$ purity.
- The method also bypasses the need for genetic manipulation although it requires a mixture of atrial and ventricular cardiomyocytes.
- Equally important is the extraordinary caution for gating of fluorescent channels amidst sorting due to a common observation of auto-fluorescence in the unstained non-cardiomyocyte populations.

NB: This method of mitochondrial labeling is highly effective for cardiomyocyte isolation. However, still newer dyes are required if one wants to isolate other cardiac cell types such as pacemaker or endocardial cells.

Aptamer Technology

- An aptamer is a single-stranded DNA or RNA sequence having a variable domain of about 40 nucleotide bases. This variable sequence is responsible for the characteristic three-dimensional structures of aptamers besides a potential

ligand-binding ability. Such attributes of aptamers manifest a binding affinity of aptamers with numerous binding molecules, enabling the distinguishing of targets differing even by a single functional group. Perhaps this is the reason why aptamers are sometimes also called "nucleic acid antibodies."

- The binding of aptamers exclusively enacts through electrostatic interactions, whereby variability in the control sequences manifests in their constitutional versatility.
- Target binding by an aptamer is affected by the folding manners of aptamers, the characteristic order of nucleic acids, and the composition of the matrix.
- The technology which generated aptamers initially is recognized as ***Systemic Evolution of Ligands by Exponential enrichment (SELEX)***, characterized by the sequential binding of oligonucleotides to the target molecules.
- Typical aptamers targets have evolved from simple ligands, such as the surface proteins.
- The very first aptamer was fabricated for human thrombin to even more complex ligands, placed along RBC membranes and even the whole cells.
- Albeit both DNA and RNA aptamers exhibit similar functionality, the DNA has the advantage of being nuclease resistant besides an easy-to synthesize approach, since there is no need for an extra transcription step.
- The selection of aptamers is made in vitro (in a suitable matrix), so the applications are not merely focused or confined to physiological functioning.
- It is quite a familiar task to tune the optical sensitivity of aptamers, such as being used as fluorescent labels or biotin tags.
- Ultimately, aptamer generation occurs through a well-controlled and reproducible mechanism, characterized by extremely low batch-to-batch variations.
- Besides the small and large molecules, aptamers are also capable of binding actions with whole cells, viruses, and tissues. Such a diverse range of potential targets confers multifunctional attributes to aptamers.
- Though not limited to this attribute, aptamers have the potential of replacing the antibodies in multiple traditional scale requirements (Xu et al. 2009; Quang et al. 2017).
- Uses of aptamers include ***Biomarker Discovery, Drug Discovery, Diagnostics, and Therapeutics***.

The Methodology of SELEX

- The very first description of SELEX was provided by *Tuerk and Gold* in 1990 after which the SELEX configuration has witnessed numerous modifications over the years (Tuerk and Gold 1990).

Briefly, the SELEX methodology is as follows:

As a first step, an oligonucleotide library is incubated with the target protein or cell.

The constituents of the library are $\mathbf{10^{14}}$–$\mathbf{10^{15}}$ random oligonucleotides sequences, each having **15–70** nucleotides in length.

Now target protein/cell would be bound with the target DNA/RNA.

- The respective DNA/RNA-bound target complexes are isolated from the unbound sequences.
- In the next step, the bound sequences are amplified using PCR or RT-PCR as and when needed for the intensification of specific aptamers.
- The enriched oligonucleotides pool is then incubated yet again with the target protein.
- Above steps are performed again till target explicit oligonucleotides become highly intense, elapsing **15–20** cycles on an average.
- Intensified aptamers are cloned in appropriate vectors for sequencing.
- The particular aptamer sequences are thereafter chemically made before being finally screened against the target to choose the maximum affinity aptamer(s).

Aptamer-Based Cell Detection

Post selection for whole cells or to serve as explicit biomarkers, the aptamers can be conjugated with a range of sensing probes such as **fluorophores, luminophore, nanoparticles**, and several others, for prompt detection of target cells.

Depending on the signal output, the aptamer-based assays are classified into four types, as follows:

Direct binding
Target-induced structural switch
Sandwich binding
Target-induced dissociation

Here is a brief discussion about each of these assays:

Direct Binding

This comprises one of the simplest assays having a single fluorophore or luminophore moiety conjugated with aptamers which binds directly with the target, generating a signal that is easily perceived using a compatible detector.

Target-Induced Structural Switch

In the event of a missing target, the fluorophore-conjugated DNA aptamer prevails as a partial duplex with another quencher conjugated to a random aptamer. Once the target is intercepted, the aptamer binding happens and releases the quencher from the fluorophore to generate the latter's signal.

Sandwich Binding

This binding mode is characterized by the immobilization of definite unlabeled aptamers over a solid phase, getting hold of the target cells. Subsequently, the biotinylated aptamers (the similar version) are added. The assembly generates the signal on subsequent streptavidin-conjugated horse radish peroxidase (HRP) binding with the biotin molecules, as in ELISA.

Target-Induced Dissociation

This assay employs DNA aptamers functionalized with gold nanoparticles (Au NPs) in a coiled manner. Once the target is promptly recognized by the coiled aptamers, the aptamers uncoil, bind with the target cells, and release the Au NPs. The as-released NPs are precipitated in a salt solution before being visualized via concurrent color variation.

Benefits and Cautions of Using Aptamers

Contrary to the antibody primed isolation assays, the aptamers are biosensing agents, and separating moieties exhibit numerous advantages, some of which are as follows:

- Preparation of aptamers is an entirely in vitro process, no longer needing the live animals and highly sensitive hybridoma cultures that are needed to generate monoclonal antibodies (mAbs).
- There is a significant diversity of aptamers that can be synthesized for targeting a particular kind of cells.
- Chemical synthesis of aptamers is relatively better controlled, scaled up using PCR, and cost-effective.
- The aptamer assays are in general very reproducible with low variability between two batches.

Restrictions of Aptamers

- Due to a large population of generated molecules, aptamers invariably exhibit a risk of non-specific binding. As a result, a lot of clones require the initial screening (for specificity), making the process elaborate and time-taking.
- Almost invariably, the cell yield is precariously low, making the method a non-suited option for rare cell isolation.
- Owing to the unaddressed safety and biocompatibility concerns to date, the use of aptamers for clinical applications is not yet approved till date.

Using the Aptamer Technology in Cell Biology

- Several cancer cells (including pancreatic, colon, breast, prostate, and glioblastoma) have been screened and scanned using DNA aptamers.
- The dearth of distinctive surface biomarkers for screening rare cells, including **stem cells, cancer stem cells (CSCs),** and **circulating tumor cells (CTCs),** renders the characterization cumbersome. Aptamers manifest a significant possibility of being successful in separating such specialized cell isolations.
- A major reason for aptamer generation involves the separation of embryonic stem cells (ESCs) from differentiated cells, mesenchymal stromal cells, and multiple immune cells.
- Table 4 distinguishes the various cell isolation methods, comprising their working principles, yield, and purity extents.

Table 4 Summary of various cell isolation techniques

Technique	Principle	Positive/ negative	Purity	Yield
FACS	Binding of surface antigen/ protein	Positive	High	Low
Aptamer binding	Binding of surface antigen/ protein	Positive	High	Low
MACS	Binding of surface antigen/ protein	Both	High	Medium
Selective growth/ culture	Physiology	Negative	Medium/ high	Low/ medium
LCM	Morphology	Positive	High	Low
RBC rosetting	Characteristic size and surface antigen/protein	Both	High	Medium
Immuno-LCM	Morphology + surface antigen	Positive	High	Low
Plastic adhesion	Surface charge and cell adhesion	Both	Low	High
Density gradient centrifugation	The density of the cells	Positive	Low	High
Filtration	Cell size	Positive	Low	High

4.5 Cell Isolation Based on a Combination of Techniques

A combination technique of cell isolation and purification fulfills two advantages:

- Combining advantages of both techniques.
- Overcoming limitations of one method with the help of another approach.

Here is an example of some combination-based cell isolation techniques:

4.5.1 Immunogenicity-Induced Cell Separation: Erythrocyte Rosetting

- Erythrocyte Rosetting (E-Rosetting) is the typical trend, wherein the RBCs are configured in the vicinity of a central cell, forming a flower-like cluster. The generation of this pattern is attributed to the characteristic binding between the ligand (positioned on the central cell) and the corresponding RBC receptor. The most well-suited illustration of this arrangement is the binding of T-cell surface antigen (protein) CD2 with the sugar-like LFA-3 homolog over the sheep RBC surface, paving way for agglutination.
- The target cells reflect an increment in the density after rosette generation, which can afterward be separated using sedimentation or density gradient centrifugation.
- The specificity and sensitivity of this molecular recognition technique could be improvised *by* including an antigen–antibody binding assay, widening the range of different cells that could adapt well in terms of being targeted and purified.

- Immuno-Rosetting can be tailored well concerning both positive and negative isolation modules.
- In the event, that a negative selection is aimed for by an enrichment cocktail, the undesired and pelleted cells are abandoned, while the desired ones could be retrieved from the gradient medium–plasma interface.
- In case the selection is positive, the desired cells prevail within the pellet; as a result of this, the RBC lysis needs to be done for separating the target cells.
- This technique of cell separation is a speedy process with practical robustness, offering a high yield to enable the unswerving separation of cells from the blood.
- A sole concern with this method pertains to the difficulty encountered in the implicit separation of various populations from the blood. FACS and MACS are, nevertheless, preferred methods for multiple separation attempts (Strelkauskas et al. 1975).

4.5.2 Immuno-Laser Capture Microdissection System

The laser beam-driven microdissection of specific cells from stained or unstained tissue sections has been well discussed earlier. LCM working accuracy could be further improvised through the use of immuno-stained sections. This allows an efficient cell capture, not merely based on morphology and implicit tissue sectioning but also based on a specific immuno-phenotype. To accomplish this, a robust immuno-staining triggered tissue sectioning, specifically for frozen tissues is in practice. In this method, following staining, microdissection, and downstream assays are worked out (Fend et al. 2000).

This relatively new method is a kind of modified immunohistochemistry-based assay for conducting immuno-LCM, which is significantly distinct from the conventional immunological staining assays. Following are the comparative characteristic attributes:

- Post sectioning, as early as possible, the tissue sections are immobilized in a cold methanol or acetone solution.
- Staining using primary and secondary antibodies is performed ideally for 90–120 s.
- The manipulations attempt to avoid any sort of section drying via the staining process forbidden sectional detachment amidst the laser scanning.
- Immuno-LCM comprises a rapid and high-throughput technique for separating multiple cell types from a tissue, based on unique immuno-phenotype traits.

Thereby, the immuno-LCM comprises an amicable methodology to analyze the followings:

- The various tumor cells in complex malignant tissues, and
- Stem cell niches in terminally differentiated tissues.

4.5.3 Cell Isolation Based on Microfluidics

- This approach works via manipulating the fluids on a micrometer scale.
- The method is well versed in clinical and biomedical applications and familiar with the terminology of **lab-on-a-chip (LOC) assay**.

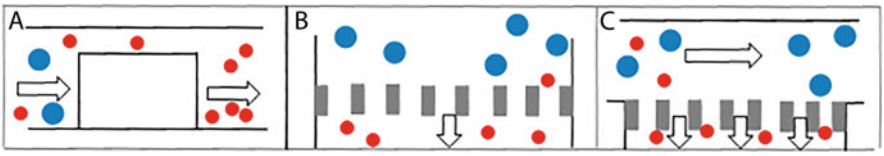

Fig. 6 Microfluidic filtration mechanisms, (A) weir filter, (B) pillar filter, and (C) cross-flow filter to separate smaller cells (in red) from larger cells (in blue)

- The method briefly involves, loading the typical cell preparations over the microchips before influenced by an external force. This separates the distinct cell populations as a function of characteristic physical and biochemical attributes.
- Functional compartments of typical microfluidic cell sorters include micro-fabricated channels, chambers, and valves.
- Figure 6 (above) depicts the microfluidic filtration mechanisms using filters in different configurations (weir, pillar, and cross-flow network) to separate the cells of higher mass from those of lower in a mixed-cell population.

Contrary to the frequently used cell sorting provisions, like automotive FACS and MACS, microfluidic cell sorting is equipped with the following benefits:

- Rapid sorting frequencies with higher output
- Easier operational steps, carriability with lower cost
- Fewer biohazards
- Enhanced purity of sorted samples
- Additionally, noted ultra-small measurements of microfluidic channels render it easy to manipulate cells, facilitating faster and prompt detection, befitting them for a consolidated in situ screening.
- The devices working on this principle are well compatible with joint functioning with manifold laboratory assays, much anticipated in translational biomedical and biotechnology research.
- This sorting method has a significant potential for separating highly pure cells usable in biomedical and clinical requirements.
- At present, these devices in assimilated mode with the FACS are used for separating the stem cells, lymphocytes, and CTCs.

Microfluidic sorting can be classified in two ways:

Active or passive microfluidic sorting
Labeled or un-labeled microfluidic sorting

Here is a brief discussion about these.

Active or Passive Microfluidic Sorting

- External force fields like an electric or magnetic field, are used for active cell sorting.
- Passive sorting depends on cell mass or density and requires either gravity or some mechanical force to sort out different cells.

Labeled or Un-labeled Microfluidic Sorting

The cells can also be sorted based on their inherent properties, for example, the hemoglobin content conferred inherent paramagnetic sensitivity, used for RBCs separation from other cells, under the influence of an external magnetic field. Yet another approach involves the use of antibodies tagged with chromophores for perceiving their binding with targeted tumor cell surface antigens. Typical methods of this regime include the microscale versions of FACS and MACS techniques. Table 5 presents a summary of microfluidic cell sorting methods.

4.5.4 Hydrodynamic Cell Sorting

- This method of cell sorting works through the force generated by fluids and is employed in numerous microfluidic devices for sorting the cells based on their size.
- The devices working on this principle comprise straight, spiral, or curved micro-channels. The cell suspension is injected into these provisions after which it is fractionated due to the inertial and frictional forces, generated in course of fluid movement.

Table 5 Summary of cell-isolating microfluidic methods

Method	Working principle	Target cells	Specific benefits
Hydrodynamic	Equivalence of elevating and frictional forces	WBCs, RBCs, and cancer cells	Separation of large-cell volumes
Acoustic	Primary acoustic radiation force	WBCs, RBCs, and platelets	Operates in non-contact mode, so less damaging to cell membranes, gives high throughput
Electrophoretic	Moving charged cells under the influence of an electric field	Movement of charged cells in an electric field	Non-contact – Can separate live and dead cells
Magnetophoretic	Movement of magnetically susceptible cells in a magnetic field	RBCs and cancer cells	Non-contact – High throughput
Optical	Optical scattering forces	Yeast cells	High resolution
Microfiltration	Size-dependent filtration across micropores	Blood cells, cancer cells	High throughput

- As the liquid material moves past the cell surface (in addition to the channel walls), it enacts an inertial elevation on the cells which is balanced by the oppositely directed friction (or drag, in the case of fluids) while moving across the surrounding fluid.
- The elevation (or lift) and drag force components are produced and varied on account of alternative contraction and expansion encountered by the channel diameters.
- The higher the cell size, the greater the lift or elevation generated amidst the cell movement. As a result, the cells of varying sizes exhibit distinctive flow trajectories when under the influence of graded hydrodynamics and movement in different channels.

4.5.5 Acoustically Driven Cell Sorting

Acoustophoresis implies the motion of an object under the typical effect of an ultrasonic wave-produced acoustic pressure difference. The development of acoustic microfluidic devices has been especially intended for spatiotemporal handling of cells as per the explicit sizes, density, and fluorescence. The technique uses one of the following acoustic wave regimes.

Bulk Acoustic Standing Waves

These waves are generated when a microfluidic channel is stimulated by the ultrasound to an extent where subjected wavelength equals the spatial measurements of the corresponding microfluidic channel. The magnitude of this force varies directly with the cell volume while its direction depends on the typical cell and fluid densities.

Standing Surface of Acoustic Wave

The provisions active through standing surface acoustic wave (SSAW) practically generate a standing wave along the channel floor. Detection of the generated wave is made via inter-digital transducers fitted on **both channel walls**.

Traveling Acoustic Waves

These non-standing surface waves are generated at the fluid surface and move to the microfluidic cavity with the help of transducers.

4.5.6 Electrophoretic Cell Sorting

This methodology makes use of an electric field that causes cell migration due to the surface charges, either already prevailing or generated via fluorophore-conjugated antibodies. The working principle of this technique is similar to FACS as it uses charged aerosol droplets for electrostatically induced cell sorting.

The different types of electrophoretic mechanisms are as follows:

Electro-osmosis
Electrophoresis
Di-electrophoresis

Ahead is a brief description of each:

Electro-osmosis

Electro-osmotic flow refers to the specific motion of a liquid in response to an applied potential difference across a porous material. The porous morphology could be accomplished via capillary tube, membrane, microchannel, and other fluid passage media. This technique resolves the drawbacks caused by an applied continuous electric current which could produce aerosols or cytotoxic by-products like H_2O_2.

Electrophoresis

- The term **"electrophoresis"** refers to a general phenomenon that infers the movement and segregation of charged particles (ions) in the presence of an electric field.
- An electrophoresis setup comprises oppositely charged electrodes (namely, anode and cathode for positive and negative charges), commonly immersed in an electrically conducting medium, referred to as an electrolyte.
- Separation of ionic particles occurs due to the differences in their velocity, (v), a product of particle's mobility (m), and the characteristic field strength (E): $1v = \text{mE}$. The mobility, in turn, depends on explicit particle morphology, charge, and the working temperature of the method, remaining unvaried under unchanged working states.
- The conditions affecting the electrophoresis working are the rate of charge flow, potential difference, power, and other parameters like ionic strength, pH, viscosity, pore size, and several others, which are a hallmark of the medium across which the particles are moving.
- The working principle of electrophoresis involves the movement of charged cell particles in the direction of the oppositely charged electrodes, being subjected to the direct current (DC) in a uniform electric field.
- Due to the lipid periphery of the cell membrane, most of the cells are slightly negatively charged and hence move toward the anode.
- Besides, fluorescent or magnetic probes used for labeling the cells also respond to changes in electric field on account of distinct surface charges.

Di-electrophoresis

- This technique is characterized by the cell movement across a non-uniform electric field, on account of the cell's charge localization in response to applied alternating current (AC).
- A particular cell's response to AC is determined by the **electrical permeability of the cells** and not by the surface charges.
- Usually, a higher relative permeability of cells results in a greater movement toward the stronger electrical fields.
- On a microfluidic scale, electrophoretic forces are generated via electrode placement at discrete locations along the strong channels (Hu et al. 2005).

5 Single-Cell Isolation Technologies

Several studies described the objectives and usefulness of single mammalian cell isolation (Shapiro et al. 2013).

Two major aims of studying the cells, at a single-cell platform, are as follows:

- Recognizing the rare cells in a mixed-cell population
- Studying genomics, transcriptomics, and proteomics at the cellular level

NB: In hybridoma technology, single hybrid cells are cultured to produce monoclonal antibodies.

In line with the major objectives of single-cell separation being the accurate recognition and analysis of rare cells, the exclusive study domains bettered by emerging advances in single-cell techniques are oncology and stem-cell biology.

Salient utilities and recent advances are summarized ahead:

Cancer Biology

- Cancer cells are broadly characterized by their manifested genome disorganization, chronological mutations, and the generation of neoplastic clones. The generated clones exhibit characteristic patterns concerning their proliferation, invasion, and metastatic ability besides the administration of chemotherapy. Owing to these reasons, studying and monitoring the tumor cells at a single cellular platform has been highly useful for their early detection and timely treatment.
- Single-cell-level study of tumors has aided in a systematic illustration of intratumor heterogeneity apart from the screening of primary tumor cells (PTCs), circulating tumor cells (CTCs), metastatic tumor cells (MTCs), and cancer stem cells (CSCs).
- Analysis of copy number patterns and mutations on a one-to-one scale has enabled a better screening of tumor evolutionary patterns.
- Timely detection and accurate sequencing of explicit CTCs have significantly enhanced our analysis of metastasis concerning tumor growth patterns and progressive gradations of the typical hallmarks.
- Last but indeed not least, combinatorial studies based on single-cell isolation, proteomics, clonal expression, and in vivo transplantation have been exclusively helpful in identifying the relapse and metastasis encouraging CSCs. Suited examples herein are colorectal, breast, and blood tumors.

Stem-Cell Biology

- The primitive intent of single-cell techniques is the separation of rare stem cells from mixed populations and manifolds their usability in autologous regenerative therapies. To recall, stem cells are those undifferentiated cells that have an ability of self-renewal and a totipotent status concerning differentiating into specific cell types.

- Isolation, culture, and in vivo transplantation of single cells have improved the recognition of stem cells against the less potent progenitors; the best suited is the hematopoietic system.
- Single-cell proteomics can enable the screening of novel biomarkers from rare adult stem cells, another means for accurate characterization and expansion analysis.
- Single-cell studies have also demonstrated the heterogeneous sensitivity of stem cells, like in neural stem cells.

Working Mechanism Behind Stem-Cell Isolation

- The basis for separating single cells is nearly similar to that of general cell isolation, wherein characteristic features of the explicit cell type are screened to distinctly recognize and segregate them from a mixed-cell population.
- In general, the properties or features that are identified include the physical texture such as size, density, and electrical charge-mediated isolation of a particular protein.
- Like the conventional cell isolation methods, single-cell isolation protocols should also be standardized for high throughput, target cell output, and relative purity.
- The terminology distinctions herein define a **limiting dilution** procedure through which either single or some more cells (one to four) are isolated via serial dilution of cell suspension.
- The method is usually more common in microbiological and mammalian cell inspection for generating monoclonal cultures. A major reason for choosing this method is the inability of conventional micro-pipetting to aliquot the cells at low densities.
- As opposed to the above, serial limiting dilution enhances the possibility of obtaining a single cell in the aliquot, in accord with ***Poisson's distribution***.

5.1 Techniques Used for Single-Cell Isolation

The following techniques are used for single-cell isolation:

Single-cell fluorescence-activated cell sorting technology
Single-cell laser capture microdissection technology
Manual cell picking using micromanipulators
Microfluidics
Droplet-based devices
Pneumatic membrane valves
Optical tweezers

For single-cell sorting and cloning, readers are suggested to go through the year 2000 work of ***Battye and colleagues*** (2000).

Here is a brief description of them:

Single-Cell Fluorescence-Driven Cell Sorting

- Isolating single cells contrary to that of particular populations based on fluorescence ability works on FACS similar methods and principles, wherein merely the end-stage product is different.
- Rather than gathering the cells in tubes, it is preferable to dispense the single cells in multi-well plates or mini-tubes having the capacity of one cell per well or per tube.
- The instrumental specifications needed to be incorporated in a standard sorting device are of two major types, as below.
- Robust harvesting platforms facilitate the optimum fitting of FACS tubes, microtubes, 384-, 96-, 24-, and 6-point graded well plates, and calibrated slides.
- The accuracy of a typical single-cell sort is maintained by an inbuilt Computerized Cell Deposition Unit (CCDU), which positions the cells in an X-Y coordinate system (Ohnuma et al. 2006).

Laser Capture Microdissection for Single Cells

- The illustration of LCM has been made earlier in this chapter.
- Fundamental LCM methodology can be used for separating the single cells from the solid tissues using the *Zeiss PALM Micro-Beam* system. This provision uses a contact-free laser pressure catapult (LPC) to detain the cells.
- To begin with, the local plasma is ignited using a defocused infrared laser stimulus beneath the earlier cut cell. Subsequently, the plasma impulse projects the cell against gravity into an appropriate collector.
- The precision can be further improvised, as also illustrated by Zeiss's initiative of amalgamating the PALM Micro-Beam system with the optical tweezer (PALM Micro-Tweezers). Instruments conferring a high precision employ a focused laser beam for generating a force field, recognized as the optical trap. The provision enables the movement of microparticles including the cells, as per the explicit dielectric attributes of the cell.
- A significant attempt herein by *Gross and associates* demonstrates the various techniques for single-cell separation (Gross et al. 2015).

Micromanipulator-Assisted Manual Cell Separation

- In general, micromanipulators comprise an inverted microscope having ultra-thin glass capillaries fitted on a motorized stage.
- These capillaries are modified to function as micro-pipettes, in turn, connected with an aspiration and release unit.
- A typical cell sample is obtained as a single-cell suspension in a well plate of optimized dimensions, after which it is placed under an eyepiece. Certain explicit cells are chosen via visual inspection, and gradually, each cell is pulled up through the capillary using suction.
- Present-day microfluidic devices have reduced the conventional cell sorting provisions to the microscopic order.

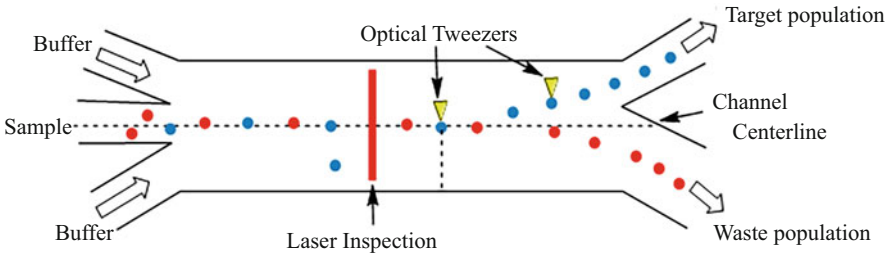

Fig. 7 Optical tweezers-based microfluidic channels for isolating the target cells

Prominently, three microfluidic devices are known, all of which are employed in synergy with FACS/MACS. The modules are successfully optimized for single-cell isolation:

Droplet-based devices
Pneumatic membrane valves
Optical tweezers

Here is a brief discussion about them:

Devices Working Through Droplets
These devices utilize the oil-filled channels that trap an aqueous droplet before being isolated. Typical chances of this droplet being contained in a cell are determined using Poisson's distribution, as in the limiting dilution method.

Membrane Valves Working Through Pneumatic Provision
Such devices use compressed air to induce motions in an elastomeric membrane (typically, the valves) which enclose the microfluidic channels. The channels are opened and closed by the air pressure-driven membrane deflection. A cell screening stimulus (such as fluorescence, electric charge, and others) controls the pneumatic action and on being detected, air pressure-driven cell movement opens the valves and allows the cell to enter the fluidic channel.

Optical Tweezers
These devices work through a microfluidic mechanism, mediated via fitted provisions in conjugation with a charge-coupled device (CCD), commencing by target-cell-captured images (identified by morphology or immuno-staining).

Henceforth, optical tweezers are used to move the targeted cells with high accuracy over a suited channel or trap (Petersson et al. 2007; Kang et al. 2012; Shields et al. 2015; Du et al. 2016). Figure 7 (above) depicts the isolation of a target cell population from an input sample using a laser probe.

5.2 Analysis Tools for Single Cells

Separation of single cells needs precise interfacing and conjugation with sophisticated screening tools specifically optimized for a single cell (Strelkauskas et al. 1975; Sydor and Nock 2003; Szaniszlo et al. 2004; Merrick et al. 2011).

The four downstream assays performed with single cells are as follows:

Single-cell genomics
Single-cell transcriptomics
Single-cell proteomics
Single-cell printing

Here is a brief description of these:

5.2.1 Single-Cell Genomics

- **A genome** is a haploid set of chromosomes (DNA) in each cell of a multicellular organism or a gamete or microorganism.
- Genome describes the entire genetic material present in a cell.
- Genomics is a specialized domain of molecular biology that deals with structure–function evolution and gene mapping.
- A formidable challenge in single-cell genome sequencing pertains to a very low quantity (~6 pg/cell) of available genomic material. Amplification of so small DNA contents using the conventional PCR approach manifests in either of the ahead errors.

Allelic Dropout
This refers to the randomized non-amplification of one of the alleles in a heterozygous model. This is a frequent bottleneck encountered in single-cell genotyping and often culminates in wrongful diagnosis.

Preferential Amplification
This constraint of single-cell genome sequencing results in accidental excessive amplification of one of the alleles compared to the other.
These constraints of single-cell genome amplification are addressed by recently developed two assays, namely:

Multiple Displacement Amplification
This is a non-PCR amplification technique, requiring a high-fidelity DNA polymerase, such as bacteriophage 29 polymerase and hexamer primers. Amplification in this protocol happens at an unchanged temperature of 30 °C, generating large sized product with a low error frequency.

Multiple Annealing and Looping-Based Amplification Cycles

This approach comprises a linear amplification assay and makes use of special primers to facilitate the looping of the amplicons. The method rules out any sort of exponential DNA formation along with the amplification bias.

5.2.2 Single-Cell Transcriptomics

- A significant constraint in the sequencing of single-cell genomes is the very low availability of genomic material. The amplification of this many DNA counts (~6 pg/cell) using PCR methods is likely to yield error-prone or inadequate outcomes.
- Transcriptome constitutes the set of entire RNA molecules in a given cell population.
- **Transcriptomics comprises the** techniques for studying an organism's transcriptome, the sum of all of its RNA transcripts in a cell.
- Typical analysis of single-cell transcriptome is done using RNA sequencing rather than cDNA microarray as the former enables a higher sensitivity and needs low sample content.

The following three steps encompass the understanding of RNA sequencing:

1. RNA reverse transcription into first-strand cDNA
2. Second-strand synthesis and cDNA amplification
3. cDNA sequencing

Two major amplification modules are used:

SMART-SEQ

The switching mechanism at the 5′ ends of the RNA transcript **(SMART)** is a PCR-based amplification method using **M-MuLV** reverse transcriptase **(RT)**. The RT adds three to four cytosine residues to the 3′ ends of the first cDNA strand which then attaches to a universal PCR primer. In this manner, the SMART-SEQ amplification module ensures the strand specificity and amplification of only the full-length transcripts.

In Vitro Transcription

In vitro *transcription* (IVT) involves the template-driven synthesis of RNA oligonucleotides that vary in length from being short to several kb. This method normally uses the T7 *Coli phage* RNA polymerase and needs a template that includes the promoter for T7 RNA polymerase, upstream of the target sequence. Contrary to the PCR methods, IVT exhibits a higher specificity and fidelity.

5.2.3 Single-Cell Proteomics

- **Proteome** refers to the complete set of proteins, generated or modified by an organism.

- **Proteomics** thereby infers a large-scale study of proteomes.
- A formidable challenge of single-cell proteomics is the same as that for single-cell genomics and transcriptomics, that is, very low biological material available for analysis.
- Furthermore, like the nucleic acids, there is no way out yet available for amplifying the proteins.
- As a consequence, traditional methods of protein analysis, like gel electrophoresis, immunoassays, chromatography, and mass spectrometry, are no longer considered under the umbrella of single-cell proteomics.
- Recently, attempts for integrating microfluidic devices (for single-cell isolation) with manifold protein assays on miniaturized platforms have been initiated.

Examples of such attempts are as follows:

Single-cell mass cytometry
Single-cell ELISA on antibody-coated microchips
Single-cell western blots

5.2.4 Single-Cell Printing

- Almost, over the entire past decade, the focus of biomedical research has steadily shifted from studying cell populations to single-cell analysis. This paradigm change of opinion has encouraged the research for developing highly precise and throughput technologies intended for isolation, culture, and omics analysis of single cells. Inevitably, a need to miniaturize and integrate cell isolation procedures with several other assays has created an interest in the development and engineering of microfluidic systems. Though LOC assays harbor a high precision, speed, and robustness of operation, no single device is capable to cater to multiple functional needs.
- A remedy to this constraint is the development of a single-cell printer (SCP), which typically combines an inkjet printer (working on the same principle) with an optical setup for the detection and printing of single living cells. Procedurally, this device works by capturing the cells in a pico-liter droplet which is thereafter deposited by the printer at designated loci for downstream analysis.
- Typical characteristics making this technique more advanced than the remaining single-cell manipulation methods include the spatial flexibility, provision of deposition, and printing of the cells on multiple surfaces like a micro-well, microscope slides, micro-tubes, etc. in a single experiment (Gross et al. 2013; Riba et al. 2016).

The following instrumentation system is highly important for single-cell printing.

The micro-dispensing chip
The vision system
The micro-pneumatic shutter
The robotic stage

Ahead is a brief discussion about them:

The Micro-Dispensing Chip

The dispenser chip in this technique is a silicon and glass micro-fabricated chamber, in which the cell suspension is loaded from the sample reservoir via capillary flow. The Si membrane of the chip is linked with the piezoelectric actuator which progressively replaces the liquid within the chamber. This activity forms and ejects the droplets from the chip nozzle. Based on specific nozzle diameter 10–100 μm, the droplets range within 100–250 pl in volume (Emmert-Buck et al. 1996).

The Vision System

This unit comprises a very receptive camera with a spatial resolution of 0.8 μm/pixel. This camera is targeted onto the dispenser chip nozzle and records a photograph before each droplet formation. Gradually, the system-designated algorithm extorts the specific position of each cell and estimates the cell number in each droplet.

The Micro-Pneumatic Shutter

For isolating the single cells, each droplet printed over the substrate must comprise a single cell. Disappointingly, it is not feasible to maintain the cell number per droplet. Owing to a random distribution of cells within a droplet, the system sorts out single-cell holding droplets from those having no or several cells. The micro-pneumatic shutter placed exactly below the chip's nozzle works in synergy with the imaging system. This facilitates only the single cell containing droplets to pass through and eliminates the others in a waste chamber.

The Robotic Stage

- The droplet generator, vision module, and pneumatic shutter, all are conjugated in a compressed "print-head" build-up over a robotic stage.
- The movable stage is controlled around the three axes, being placed relative to the print-head for getting the cells printed over several kinds of substrates like slides, micro-wells, PCR tubes, etc.
- The method of single-cell printing (SCP) operates with good speed, precision, and flexibility besides exhibiting cost-effectiveness. A constant incentive herein is its label-free isolation procedure.
- SCP technology has numerous applications which can significantly improve our understanding of cell and molecular biology on the single-cell platform.
- SCP is very well accustomed for its usefulness in molecular and genetic characterization of implicit tumor cells, imparting significant insights into clonal heterogeneity.
- This method can be modified to analyze and screen the stem cells, monitor the various aspects of developmental biology, lineage mapping, and a range of other applications based on single-cell scrutiny.

6 Conclusions

A mammalian body consists of trillions of cells. These cells are attached in various proportions to form different tissues and organs that collectively constitute a complete mammalian body. Initially, it needs to be disintegrated for dissociating the cells from the tissues or organs. Once cells are dissociated, they can be isolated based on their shape, size, morphology, and other structural and functional characteristics. This chapter described all these separation techniques along with their pros and cons. Finally, in today's scientific world, isolation of single cells fulfills two purposes: (1) Identification of rare cells in a heterogeneous population, (2) Understanding Genomics, Transcriptomics, and Proteomics at the level of the basic biological unit, ***"The Cell."*** Besides describing various single-cell isolation technologies, this chapter also focused on various tools for single-cell analysis such as genomics, transcriptomic, and proteomics. This chapter will, therefore, enhance the reader's knowledge regarding the isolation and purification techniques of mammalian cells, including single cells.

7 Cross-References

- ▶ Culture of Continuous Cell Lines
- ▶ Culture of Neuron and Glia Cells
- ▶ Isolation and Primary Culture of Various Mammalian Cells
- ▶ Mammalian Cell Culture: An Overview
- ▶ Mammalian Cell Culture Types and Guidelines of Their Maintenance
- ▶ Primary Culture of Immunological Cells
- ▶ Stem Cell Culture and Its Applications

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Isolation and Primary Culture of Various Mammalian Cells

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Contents

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_8-1

Abstract

Isolation and primary culture of four major mammalian cells comprise the major contents of this chapter. The cells described here are endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. While endothelial cells are present only in the various micro- and macrovascular beds, smooth muscle cells and fibroblasts are present both in the vascular and nonvascular regions of a mammalian body. However, epithelial cells are present in the epithelium, located exclusively in the nonvascular regions. The epithelium is the thin tissue layer or

multiple layers of epithelial cells, forming the inner or outer surface of organs, glands, mouth, nostrils, trachea, alveoli, ducts lumen of mammary glands, the lining of the alimentary canal, and urinary bladder. This chapter focuses on the following basic aspects of the isolation and culture of these mammalian cells: (1) the basic concept of these cells; (2) locations of the body at which these cells are present; (3) various instruments and materials are needed to isolate these cells; (4) various isolation and culture procedures of these cells; (5) phenotypic and protein maker-based identification of these cells; and finally, (6) utility to culture prospects of these cells.

Keywords

Isolation of cells from tissue/organs · Importance of Mammalian cell primary culture · Primary culture of cells · Endothelial cells · Vascular smooth muscle cells · Fibroblasts · Epithelial cells · Phenotypic identification of cells · Marker protein-based identification of cells

1 Introduction

This chapter describes the isolation and primary culture of various mammalian cells. Cells for primary culture originate directly from organs and are recovered either via mechanical disruption or enzymatic dissociation. Access to fresh mammalian organs or organ-derived tissues is required for isolation and primary culture of cells. A major benefit of primary cultures is that the cells maintain most of their innate characteristics and normal physiological functions, particularly during early passages. The basic approach for isolation, characterization, and primary culture of vascular endothelial cells, vascular smooth muscle cells, vascular and nonvascular fibroblasts, and epithelial cells is described in this chapter. While large blood vessels are made up of endothelial cells (in the tunica intima), smooth muscle cells and fibroblasts prevail in the tunica media and tunica adventitia, respectively, epithelial cells are present particularly in the lining organs of mammals. Besides epithelial cells, smooth muscle cells and fibroblasts are exclusively present in the nonvascular regions of the body.

The following points focused on describing the isolation and culture of each of the abovementioned cell types: a basic concept of each of these cells, that is, endothelial, smooth muscle, fibroblasts, and epithelial cells; the major vascular and nonvascular regions of the mammalian body at which these cells are located; various instruments and other materials necessary for the isolation and culture of these cells; phenotypic and other marker protein-dependent identification of these cells; different culture procedures of these cells as described by various scientists; and finally, utility of culturing these cells. The content of this chapter would prove a valuable database for strengthening an understanding of the isolation and culture of mammalian cells, both from vascular and nonvascular origin.

2 Major Types of Mammalian Cells Used in Primary Culture

For isolation of mammalian cells, fresh organs and tissues are collected. Generally, cells are isolated from various tissues and organs either by enzymatic or mechanical dissociation. Following isolation from various tissues and organs, cells are subjected to culture in a CO_2 incubator.

In this chapter, the isolation and (primary) culture of the following cells are discussed:

Isolation and primary culture of vascular endothelial cells
Isolation and primary culture of vascular smooth muscle cells
Isolation and primary culture of vascular and nonvascular fibroblasts
Isolation and primary culture of epithelial cells

3 Isolation and Primary Culture of Vascular Endothelial Cells

3.1 Endothelial Cells

- Endothelial cells are a thin layer of squamous cells forming the interior surface (lumen) of blood vessels (artery–capillary–vein), lymphatic vessels, bone marrow, and the interior surface of the heart (endocardium).
- Endothelial cells originate from epithelial cells, which in turn are of mesodermal origin.
- The term **endothelium** refers to endothelial cells that line the interior surface of blood vessels (vascular endothelial cells) and lymphatic vessels (lymphatic endothelial cells), respectively.
- The mammalian vascular bed consists of **microvessels** and **macrovessels**. While microvessels are located in the capillary bed, macrovessels are the major blood circulation tubes of the mammalian body. The macrovessels are involved in various functions, including the blood–brain barrier, clearance of sinusoidal endothelial cells in the liver, and blood filtration by glomerular vascular segments in the kidney. **The inner lining of both micro- and macrovessels is made up of a single layer of endothelial cells.**
- The entire vascular bed is made up of a single endothelial cell layer.

3.1.1 Locations of Endothelial Cells in Humans

- A generic endothelial cell does not exist.
- Endothelial cells differ in structure and phenotype, depending on the vessel type.
- In microvessels (generally, (3–4) μM in diameter) a single layer of endothelial cells is supported by a basal lamina.
- However, macrovessels (up to 25 mm in diameter) consist of three cell layers, namely,
- **Tunica intima**

  Endothelial and pericytes cell layer lining the inside of vessels.

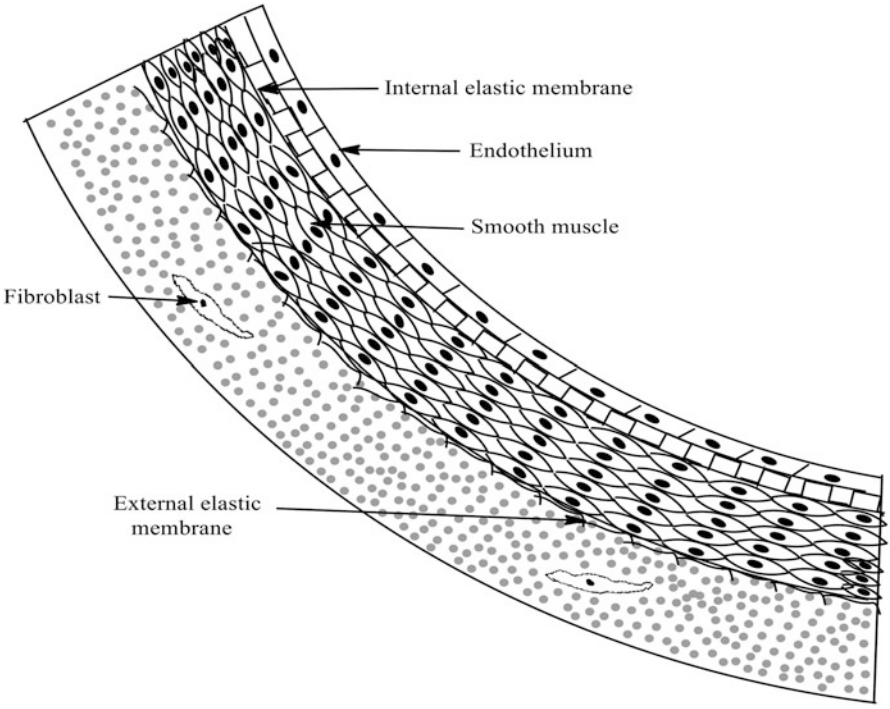

Fig. 1 Pictorial depiction of various layers of a large vessel (e.g., artery)

- **Tunica media**
  The smooth muscle cell layer forms the middle layer of large blood vessel walls.
- **Tunica adventitia**
  The outer thickest layer of large blood vessels is constituted of fibroblasts.
  Other components of the tunica adventitia are mast cells, nerve endings, vasa vasorum, and collagen fiber **(**Fig. 1**)**.

3.2 Dissecting Instruments for Isolating Endothelial Cells

Scissors, forceps, scalpels, syringes (10 and 20 ml), a small cannula or plastic tube, and a 20 cc syringe are needed for dissection and collection of endothelial cells from blood vessels such as an umbilical vein. Before use, the sterility of all materials must be ensured.

3.3 Materials for Culturing Isolated Endothelial Cells

3.3.1 Specimen Collection Container

Sterile plastic containers are used to collect blood vessels/umbilical cords.

3.3.2 Cell Dissociation Enzyme

The freshly prepared enzyme (e.g., 0.1% filter-sterilized collagenase is used to isolate HUVECs).

3.3.3 Buffers

Sterile phosphate-buffered saline (PBS) without Ca^{+2} or Mg^{+2} is used to wash the umbilical cord or the lumens of other blood vessels.

3.3.4 Cell Culture Medium

The culture configuration for endothelial cells: M199 with Earl's salt or Dulbecco's Modified Eagle's Medium (DMEM). To make a complete medium, the following ingredients need to be added: (5–10)% fetal calf serum (FCS) or fetal bovine serum (FBS), 100 $U \cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycins, 100 $\mu g \cdot ml^{-1}$ heparin sodium salt, (10–50) $\mu g \cdot ml^{-1}$ vascular endothelial cell growth factor (VEGF), and 2 mM L-glutamine (for faster growth). Make a complete medium, filter-sterilize the aliquot, and store at 4 °C for use within a week.

3.3.5 Cell Adhesive Agents

Sterile 0.1% gelatin (cheapest adhesive agent). Laminin or poly-L-lysine can also be used.

3.3.6 Cell Culture Containers and Associated Materials

Flasks ((25 or 75) mm) or plates ((30, 60, or 100) mm), (15 and 50) ml centrifuge tubes, and pipettes of various capacities are needed for cell culture. All these materials should be sterile, disposable, of mammalian cell culture grade, and made using polystyrene. Membrane filters having various pore sizes are used for filter sterilization of the culture medium.

3.3.7 Blood Vessels

All blood vessels, macro or micro, can be used for the isolation and culture of endothelial cells.

3.4 Cell Division Capacity and Growth Properties of Endothelial Cells

- Endothelial cells are characteristically "quiescent" as they do not actively proliferate. The average lifespan is >1 year.

- A fine illustration pertains to distinct turnover times in different organs, a couple of months for liver and lung, while for brain and muscle, this duration lasts for years.
- However, in embryonic tissues, damaged blood vessels, and menstruating uterus, the endothelial cell doubling time is only a couple of days. So, it is possible to culture the endothelial cells, in vitro.
- A characteristic of in vitro cultured endothelial cells is the (48–72) hour doubling time during the logarithmic growth phase.
- Microvascular endothelial cells isolated from the retina of the macaque monkey exhibit a mean doubling time of 44.5 h during the first (3–5) days of culture and 23 h at (6–8) days of culture, forming a confluent monolayer within (12–14) days.

NB: A 75 mm flask with 1 × 10^6 cells takes (2–3) days to reach 90% confluence. An endothelial cell may divide for about 50 passages, following which cells undergo apoptosis.

3.5 Types of Blood Vessels in Humans

Based on the diameter, human blood vessels are divided into the overleaf two general groups:

Macro blood vessels
Micro blood vessels

The following is a brief discussion of these vessels and the endothelial cells present in them:

3.5.1 Macro Blood Vessels

These include the following:

Coronary artery/vein
Pulmonary artery/vein
Aorta and large arteries/veins present in the brain and limbs

3.5.2 Micro Blood Vessels

These are the blood vessels (such as **capillary, arteriole, or venule)** of the micro-circulatory system. In mammals, each organ has its blood vessels and vascular bed.

Arterioles are the typical, small-diameter blood vessels that extend and branch out from an artery, leading to capillaries.

Capillaries: These are perhaps the smallest blood vessels.

Metarterioles: These are the vessels linking arterioles and capillaries.

Venules: The vessels that allow deoxygenated blood to return from the capillary beds to veins.

Thoroughfare channel: A venous vessel receiving blood directly from capillary beds. It is a tributary of venules.

NB: Blood flows away from the heart to arteries, then into arterioles, which further narrow down into capillaries. Capillaries flow into venules and connect to veins, which return blood to the heart.

Endothelial cells are present in the lumen of all blood vessels and serve a very important role in the structural and functional integrity of the vessels.

Type 2 diabetes is more closely associated with "microvascular" complications (retinopathy, nephropathy, and neuropathy) and "macrovascular" complications affecting the heart, brain, and foot.

3.5.3 Some Physiological Distinctions Among the Endothelial Cells Originated from Various Vascular Beds

Endothelial cells differ in their macro- versus microvessel distribution besides the characteristic distinctions between the manifold vascular origins or even within the different portions of the same vascular bed.

Some physiological differences between various endothelial cells are as follows:

- Human microvascular endothelial cells differ from macrovascular endothelial cells in their matrix metalloproteases (MMPs) expression, bradykinin degradation (Jackson and Nguyen 1997; Gräfe et al. 1994), and plasminogen activator inhibitor-1 activity (Gräfe et al. 1994).
- Microvascular endothelial cells have a different prostaglandin and prostanoid secretion profile than their macrovascular counterparts and endocardium (Mebazaa et al. 1995).
- Endothelial cells isolated from the porcine coronary artery release more endothelin 1 (ET-1) than those from the aorta.
- Receptors for adenosine, α-thrombin, histamine, and acetylcholine appear to be much more abundant in microvascular guinea pig coronary artery endothelial cells than those of bovine aorta (Mehrke et al. 1990).
- *The cerebral vascular endothelial cells have distinctive features, which may differ from one other species. For example, humans and dogs have different vascular beds with different endothelial cell characteristics (*Gerhart et al. 1988).
- The fibrinolytic potential of cerebral and umbilical vein endothelial cells is differentially modulated by alpha thrombin (α-thrombin) (Shatos et al. 1995).
- There are structural and functional differences between pulmonary micro- and macrovascular endothelial cells (Stevens 2011).
- There are differences in cell adhesion molecule expression in micro- and macrovascular endothelial cells. Microvessel endothelial cells express a larger contingent of adhesion molecules than macrovessel endothelial cells.
- At a functional level, the homing of leukocytes to specific lymphoid and nonlymphoid tissues occurs exclusively within the microvascular compartments and is mediated by differentiation and sequential expression of specific adhesion molecules in a tissue-specific manner (Ades et al. 1992).

- In 2007, *Arid WC* in two consecutive publications described the endothelium heterogeneity, along with their structures and functions, in parallel, emphasizing the value of viewing endothelium as an integrated system (Arid 2007a, b).

3.6 Isolation and Culture of Vascular Endothelial Cells from Human Umbilical Veins

- The human umbilical cord contains two small-diameter arteries and one large-diameter vein (Gruber et al. 2021). The human umbilical vein (HUV) is the source of the simplest and most easily isolated endothelial cells and is widely used in biomedical research.
- Although HUVECs exhibit all general attributes of endothelial cells, they are of fetal and venous origin.
- One can also isolate umbilical artery endothelial cells. However, this is technically more difficult because of the smaller native diameter.
- Endothelial cells may also be isolated from many other blood vessels, including the aorta or microvessels.
- The isolation procedure was described by Jaffe et al. 1973.

Isolation of endothelial cells from the human umbilical cord vein involves the following steps.

Step 1: Collection of umbilical cord
Step 2: Washing of the umbilical cord vein lumen
Step 3: Enzymatic digestion of the umbilical cord vein lumenal surface
Step 4: Collection of endothelial cells from the digested vein
Step 5: Coating the cell culture vessels (containers) with 0.1% gelatin
Step 6: Plating and culture of endothelial cells in gelatin-coated containers

What follows is a brief discussion of the above steps:

3.6.1 Step 1: Collection of umbilical cord

The followings are the key points about umbilical cord collection:

- Collect fresh (within 1–3 h after baby delivery), (5–20)-cm-long umbilical cords.
- Place harvested cords into sterile containers containing sterile Dulbecco's phosphate buffer saline (DPBS) or Hanks' balanced salt solution (HBSS) media without Ca^{+2} and Mg^{+2}.
- Cords should be inspected and all areas with clamp marks should be excised to lessen the chances of cell contamination (e.g., smooth muscle cells/fibroblasts) from the clamp-damaged regions.
- Discard the cord if one is below 12 cm in length or the clamp marks are less than 12 cm apart.

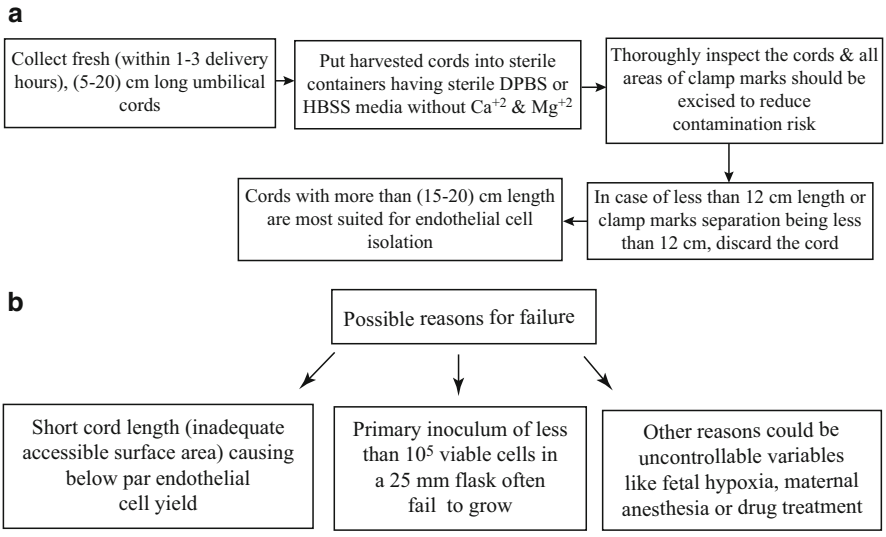

Fig. 2 (**a**) Chronological steps for harvesting the umbilical cord cells. (**b**) Possible reasons for not being able to harvest the umbilical cord cells

- In general, cords with more than (15–20) cm in length are most suitable for endothelial cell isolation.
- Cords must be processed within (1–3) hours of birth and should be discarded if the processing is delayed for more than 3 h.

NB: About one-third of the cords fail to provide viable cultures despite the use of fresh medium and all necessary additives.

- A major factor for failure is the short length of the cord (insufficient intimal surface area) leading to insufficient endothelial cell yield.
- A primary inoculum of less than **1×10^5 viable cells** in a 25 mm flask usually fails to grow.
- Figure 2(a) depicts the functional steps for harvesting the umbilical cord cells, and the possible reasons for its failure due to uncontrollable variables such as fetal hypoxia, maternal anesthesia, or drug treatments are shown in Fig. 2(b).

3.6.2 Step 2: Washing of the umbilical cord vein lumen

The over-leafed points need to be remembered regarding washing of umbilical cord vein:

- Bring the umbilical cord container into the laminar flow hood.
- Rinse the cord exterior with sterile DPBS, without Ca^{+2} and Mg^{+2}.
- **Identify the Umbilical Cord Vein**

Fig. 3 The axial view of an umbilical cord

An umbilical cord has two arteries and one vein. Arteries are smaller and thick-walled because of a thicker smooth muscle coat. The umbilical vein is substantially larger in diameter than the umbilical arteries. The umbilical vein is easily dilated (Fig.3).

- Insert a cannula or tube attached to a 20 cc syringe into one end of the umbilical vein lumen.
- Then, use this 20 cc syringe to flush the vessel with (200–300) ml sterile DPBS or HBSS for removing blood inside the vein lumen.
- Allow the vessel to drain the DPBS or HBSS by gravity from the other end.
- Do not introduce air into the lumen.

3.6.3 Step 3: Enzymatic digestion of the umbilical cord vein lumenal surface

The following details need to be adhered to during luminal digestion of an umbilical cord:

- Tie off or seal one end of the umbilical vein and leave the other end open.
- Fill a sterile syringe with 20 ml sterile prewarmed (37 °C) 0.1% collagenase solution.
- Place a syringe-connected tube into the open end of the umbilical vein before filling the lumen with 0.1% collagenase type 1 from *Clostridium histolyticum* (Gibco-BRL).
- Remove the syringe and clamp the end of the umbilical cord.
- Now both ends of the umbilical cord are sealed.
- Place the cord into a sterile container, close the container, and remove it from the laminar flow hood.
- Incubate the container at 37 °C inside a CO_2 incubator.
- Collagenase will remove the luminal endothelial cell layer of the umbilical vein.
- After (15–20) minutes of incubation in the CO_2 incubator, place the cord into a laminar flow hood. Gently massage the vein and remove the seal/clamp from one end of the vein, keeping the other inside a sterile container.
- Open the other end of the umbilical vein.
- Insert a needle of a syringe at the latter opened end of the umbilical vein before flushing the umbilical vein once with sterile DPBS into the same container that contains collagenase extract.

3.6.4 Step 4: Collection of endothelial cells from the digested vein

- Place the DPBS/collagenase-containing endothelial cells suspension into a 50 ml sterile centrifuge tube.
- Centrifuge the tube at 1000 rpm for 5 min.
- The yield in this procedure should be in the range of (0.5–1.5) $\times$ 10^5 cells.

NB: Any preparation contamination at this stage can usually be detected. A bulky gelatinous pellet indicates the presence of connective tissue matrix and cellular elements from deeper areas of the vessel wall, while the presence of red cells indicates an inadequate luminal blood flushing.

The collected umbilical cord should be used within 3 h, otherwise human umbilical vein endothelial cells (HUVECs) do not grow properly.

After the isolation, the umbilical cord and all other used materials may be treated with a 10–15% solution of bleach for 15 min before putting into biohazard containers.

3.6.5 Step 5: Coating the cell culture containers with 0.1% gelatin

- Endothelial cells are adherent by nature. While there are many mammalian cell adhesive agents available including poly-L-lysine, laminins, and fibronectin, gelatin is the cheapest, most easily available, and most useful.
- For the preparation of gelatin, add 100 mg in 100 ml of deionized water before autoclaving and cooling. This results in 0.1% gelatin. Store this gelatin stock solution in a 4 °C refrigerator.
- In the laminar flow hood, add (2–3) ml of 0.1% gelatin in a 100 mm Petri plate or (4–5) ml in a 75 mm flask. Close the container. Shake to cover the entire cell culture surface of the container. Incubate the cell culture vessel/container at 37 °C for 30 min. Gelatin will attach to the plate surface.
- Following incubation, take the container to a laminar flow hood, remove the gelatin solution, and rinse the containers with DPBS without Ca^{+2} or Mg^{+2}.
- Until use, place DPBS in the container to prevent dryness.

NB: The gelatin-coated plates can be stored up to a month at 4 °C for future use.

3.6.6 Step 6: Plating and culture of endothelial cells in the gelatin-coated containers

- Collect the cell pellet from the centrifuge and resuspend it in a 10 ml complete medium. Place it into a 0.1% gelatin-coated 100 mm Petri plate.
- Alternatively, resuspend the cells in a 15 ml complete medium and place them into a 75 mm flask.
- Incubate the cell culture container overnight in a CO_2 incubator at 37 °C in a 5% CO_2 environment.
- Henceforth, examine for cell growth using an inverted phase-contrast microscope (Fig. 4).
- At the time of plating, 90% of the harvested cells should be viable using Trypan Blue exclusion.

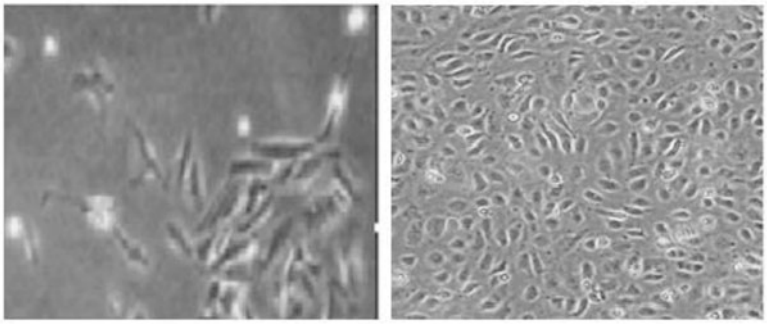

Fig. 4 The culture of endothelial cells from the human umbilical vein as observed through inverted microscope: (a) endothelial cell growth after 24 h of seeding; (b) completely confluent human umbilical vein endothelial 4–5 days after seeding

- On being 12 h in culture, nearly (25–30)% of cells become adherent. Cellular aggregates may attach to the plate as clusters.
- Change the medium to remove dead cells and allow the living cells to further expand.
- Check the growth characteristics of endothelial cells daily. The approximate morphology after 9 days of preserving in culture medium at 100% confluent stage is depicted in Fig.4.
- Harvest, subculture, and freeze HUVECs.

3.7 Limitations of Isolation of Endothelial Cells from Umbilical Cord

- The umbilical cord is of fetal origin.
- The endothelial cells isolated from the umbilical cord generally are of venous origin.

NB: Umbilical cords have one large-diameter vein and two small-diameter arteries.

3.8 Rationale for Isolation of Endothelial Cells from Umbilical Cords

- Umbilical cords are abundantly available.
- In comparison, pulmonary artery, coronary artery, and cerebral artery are available primarily when there is a need for transplantation surgery or in case of accidental death.
- Consent of the donor is essential before collecting any organ.

3.9 Isolation and Culture of Vascular Endothelial Cells from Microvessels

In 1992, ***Ades and colleagues*** generated a human dermal microvascular endothelial cell line (HMEC-1). This cell line is currently the most widely used for microvascular endothelial cell culture for diverse research requirements (Ades et al. 1992).

The following points need to be considered when culturing microvascular endothelial cells:

- During in vitro culture, microvascular endothelial cells undergo morphological differentiation into capillary-like structures much more rapidly and readily than large blood vessels.
- Loss of microenvironment-driven control when culturing endothelial cells is a major hurdle in obtaining an accurate depiction of the molecular heterogeneity of in vivo functioning.
- The isolation and culture procedures for microvascular endothelial cells are generally the same as for HUVECs.
- Several groups have described the isolation and culture of microvascular endothelial cells.

NB: In general, since microvasculature is very small in diameter it is very difficult to isolate the endothelial cells.

- The number of cells isolated from the microvasculature is very small. Moreover, growing such a small number of cells in cell culture containers is difficult due to missing cell-to-cell signaling.
- Microvascular endothelial cells are much more fastidious in growth; the in vitro culture requirements of microvascular cells are more complex than large vessel endothelial cells with a significant extent of biological differentiation and functional distinctions.
- To partially overcome these problems, *Ades and associates* have established an immortal dermal microvascular endothelial (**HMEC-1**) cell line, which is now widely used for experimental purposes (Ades et al. 1992).

3.10 Phenotypic Identification of Endothelial Cells

The following are the phenotypic characteristics of endothelial cells, as observed through an inverted microscope:

Shape of Endothelial Cells in Two-Dimensional Cultures
In general, endothelial cells appear flat having a central polygonal morphology with a cobblestone surface.
Thickness of Endothelial Cells in Two-Dimensional Cultures
Endothelial cells are (1–2) μm in thickness when cultured in two dimensions.

Diameter of Endothelial Cells in Two-Dimensional Cultures

Endothelial cells from various blood vessels (e.g., micro- versus macrovessels) exhibit a significant variation in their diameters, ranging from (3 to 20) μm.

3.11 Marker Proteins Expression-Based Identification of Endothelial Cells

As mentioned, endothelial cells are phenotypically identified by a cobblestone or polygonal morphology. During embryonic development, these cells differentiate from a common precursor called angioblast and acquire organ-specific properties. One of the important determinants of endothelial cell differentiation is the local environment and especially the interaction with surrounding cells. This interaction may occur through the release of soluble cytokines, cell-to-cell adhesion molecules, communication proteins, as well as the synthesized matrix proteins on which the endothelium adheres and grows. The acquisition and maintenance of specialized properties by endothelial cells are highly essential for the functional homeostasis of different organs. For instance, in the brain, alteration of the blood–brain barrier (***BBB***) properties may have important consequences on functional integrity. Von Willebrand factor (***VWF***), together with the Weibel–Palade bodies (***WPB***), angiotensin-converting enzyme (***ACE, CD143***), and the cobblestone morphology specific for monolayer cultures, have all been previously referred to as a few obligate criteria to confirm the authenticity and purity of cultured endothelial cells.

Here is a list of the most common endothelial cell phenotype markers. These molecules and related antibodies may be valuable tools for endothelial cell isolation and characterization (Garlanda and Dejana 1997).

Endothelial cells are screened by the presence of the following molecules:

Weibel–Palade Body

Microscopically, endothelial cell cytoplasm is characterized by the large rod-shaped organelles called Weibel–Palade bodies (WP bodies). These organelles store von Willebrand factor (blood glycoprotein involved in homeostasis) and P-selectin (the cell adhesion molecule on the surface of activated epithelial cells, lining inner surfaces of blood vessels and activated platelets).

Von Willebrand Factor

A large proportion of adhesive glycoprotein is secreted into the blood, facilitating coagulation following hemorrhage. It is also known as Factor VIII-related antigen. The deficiency of this molecule may lead to hemophilia.

CD31

A cluster of glycoproteins is expressed on the surface of endothelial cells. The protein cluster of differentiation 31 (CD31) is also known as platelet endothelial cell adhesion molecule-1 (PECAM-1).

Vascular Endothelial Cadherin

An implicit endothelial cell adhesion molecule is located at junctions between endothelial cells, also known as a CD.

Blood Group Antigens

ABO blood group antigens (proteins) are present on the surface of endothelial cells. They are not specific for endothelial cells as these are also expressed by RBC.

Acetylated Low-Density Lipoprotein

Endothelial cells take up the acetylated low-density lipoproteins (LDLs), which can also be used as endothelial cell markers (Garlanda and Dejana 1997).

3.12 Utility of Endothelial Cells

Endothelial cells are involved in the following aspects of vascular biology:

3.12.1 Barrier Function

- The endothelium acts as a semiselective barrier between the vessel lumen and surrounding tissue, controlling the constituent's passage and the transit of white blood cells in and out of the bloodstream. The passage of various molecules across the endothelium is regulated by four different arrangements of endothelial cells in the endothelium, forming four alternative structures.
- In continuous structure, the single layer of endothelial cells is a continuous arrangement in such a way that it completely encloses the vessel lumen. The only gaps are the sealed cellular locations via junctional complexes. This structural regime is most restrictive toward the diffusion of biomolecules and proteins.
- The second type of endothelium, called fenestrated endothelium, contains small holes (25 nm) that allow the diffusion of molecules and small proteins. Diaphragms across the fenestrae and the basement membrane limit the diffusion of materials. This type of endothelium is located in the intestines and kidneys.
- The third type of endothelial cells comprises a discontinuous arrangement. Larger gaps, lack of diaphragms, and altered basement membrane characterize these endothelial cells. These endothelial cells are found in the liver and spleen.
- The final limiting diffusion of molecules across the endothelium is provided by the junctional complex between endothelial cells (where one endothelial cell meets its neighbor). Junctional complexes prevent paracellular diffusion of materials.
- Figure 5 summarizes the diversity of endothelial tissue, based on their molecular transport abilities and characteristic prevalence within the body. The permeability of the endothelium is determined by its specific regime, the basement membrane, the glycocalyx, and intercellular junctions. Transcytosis is a highly selective process that regulates the movement of proteins across endothelial cells.

NB: Excessive or prolonged increments in the permeability of endothelial cell monolayer, as in the case of chronic inflammation, may lead to tissue edema/swelling.

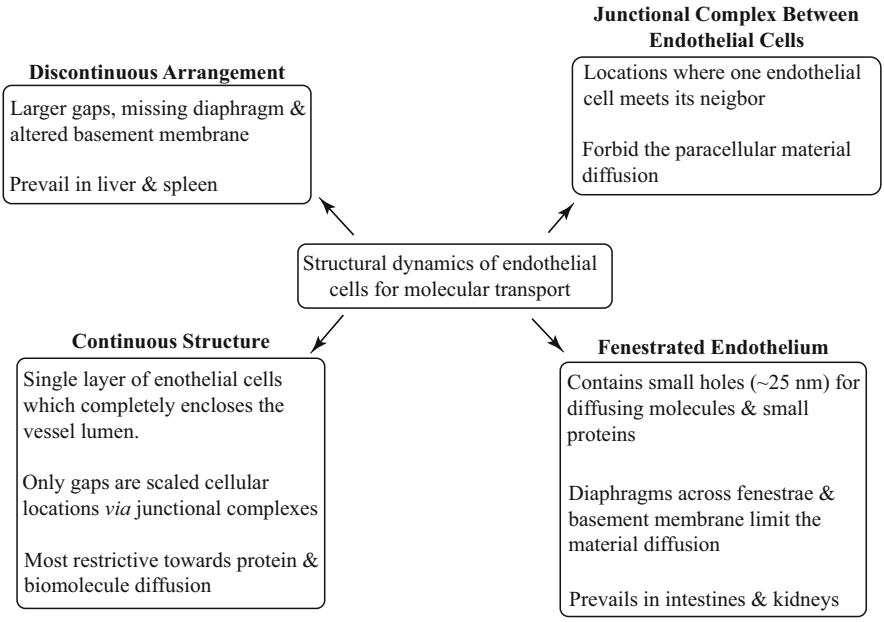

Fig. 5 Diversity of endothelial cells in terms of their molecular transport ability

3.12.2 Prevention of Clotting in the Blood Vessels

The endothelium normally provides a non-thrombogenic surface because it contains, for example, heparan sulfate (HS), a cofactor for activating antithrombin and a protease that inactivates several factors in the coagulation cascade.

3.12.3 Angiogenesis

- Angiogenesis is the typical formation of new blood vessels. Low oxygen in the blood (e.g., during tumor/cancer formation) and wounding of the endothelium (e.g., during atherosclerosis) initiate the growth of new blood vessels.
- One of the initial steps of angiogenesis is the expression of angiopoietin 2 (vascular growth factor promoting cell death through disruption of vascularization) and vascular endothelial growth factor (**VEGF**).
- Angiopoietin-2 interacts with VEGF to activate specialized endothelial cells called tip cells. Tip cells divide and invade/migrate into surrounding tissues by extending filopodia and tube formations. This leads to the formation of new blood vessels.
- Experiments in culture demonstrate that endothelial cells in a culture medium containing suitable growth factors spontaneously form the capillary tubes, even if they are isolated from other cells.
- Since capillary tubes that develop in culture do not contain blood and nothing travels through them, blood flow and pressure are not required for the initiation of a new capillary network.

3.12.4 Vascular Tone and Blood Pressure

- Endothelial cells are the source of various vasoactive agents, including nitric oxide (**NO**), prostacyclin, arterial natriuretic peptide (**ANP**), and endothelin 1 **(ET-1**).
- Other humoral agents, including angiotensin II (**Ang. II)**, aldosterone, and bradykinin, act on the endothelial cells.
- These vasoactive molecules regulate vasomotion (vasoconstriction and vasodilation) and thus control blood pressure.

3.12.5 Fluid Filtration

- Some endothelial cells are involved in fluid filtration. One example of such endothelial cells is the cells in the glomerulus of the kidney (Aird 2007a, b).

3.13 Human Diseases Related to Vascular Endothelium Defects

Since vascular endothelium controls all the biological functions of capillaries, any defect of vascular endothelium leads to multiple diseased conditions. The list of these diseases includes venous thrombosis, peripheral vascular disease, insulin resistance, diabetes, chronic kidney failures, tumors, and cancers.

- Immunological cells in the blood (e.g., macrophages) roll onto endothelial cells and attach to their surface. Attachment of blood cells (e.g., monocytes) requires a cell surface receptor, such as very late antigen 4 (**VLA4**), which binds to endothelial cell surface adhesion molecules [e.g., vascular cell adhesion molecule 1 [**VCAM-1]**).
- Following attachment, monocytes and other leukocytes transmigrate to the subendothelial space, leading to localized inflammation. Subsequently, these subendothelial leukocytes accumulate oxidized lipids and proteins inside them, leading to **fatty streak formation**. Fatty streak formation is an early stage of **atherogenesis**.
- Isolation and culture of endothelial cells serve as a useful tool for the study of atherosclerosis (fatty streak formation), arteriosclerosis (hardening of the artery) hypertension (increase in blood pressure), angiogenesis (new blood vessel formation), cancer therapy, burn therapy, wound healing and regeneration, cell signaling (e.g., **NO signaling**), gene expression profiling, drug/toxic testing and screening, and tissue engineering (Rajendran et al. 2013).

4 Isolation and Primary Culture of Vascular Smooth Muscle Cells

4.1 Vascular and Nonvascular Smooth Muscle Cells

- Smooth muscles are involuntary muscle tissues, confined within the walls of viscera and blood vessels. These are comprised of nonstriated, spindle-shaped cells.
- Based on their location, smooth muscle cells are placed into two groups, vascular and nonvascular.
- Smooth muscle cells present in the tunica media of large blood vessels (macrovessels) are called vascular smooth muscle cells (VSMCs).
- VSMCs maintain the structural integrity of macrovessels and regulate their diameter by contracting and relaxing in response to vasoactive stimuli. Thus, VSMCs help in regulating vascular tone and blood pressure.
- Smooth muscle cells (SMCs) also prevail in the walls of hollow organs, including the stomach, intestines, urinary bladder, uterus, the respiratory, urinary, and reproductive systems, and eye and skin tissue. These cells are referred to as nonvascular smooth muscle cells or simply SMCs.

NB: The intermediate filament and actin composition of VSMCs reflects a differentiation pathway, separate from other nonvascular SMCs present in other organs.

4.2 Distinctions Between Endothelial and Smooth Muscle Cells

- Smooth muscle cells constitute the **middle layer of blood vessels,** whereas endothelial cells constitute the **inner layer of a blood vessel**.
- By transmission electron microscopy (**TEM)**, cultured endothelial cells could be visualized as comprised of cytoplasmic inclusions (**Weibel–Palade bodies**). These inclusions are also found in endothelial cells lining umbilical veins but are not seen in smooth muscle cells or cultured fibroblasts, in situ.
- Cultured endothelial cells contain abundant quantities of smooth muscle actomyosin (actin–myosin).
- However, endothelial cells do not stain as intensely for actin as do the SMCs. This distinction is a preferred approach for determining the SMC's presence in disorders such as atherosclerosis.
- Cultured endothelial cells also contained ABH antigens appropriate to the tissue donor's blood type; these antigens are not detectable in cultured SMCs or fibroblasts.
- Human endothelial cells express FasL (death factor-inducing apoptosis) on the surface to a higher extent than do VSMCs.
- When subjected to variable durations of supercooling and rewarming, SMCs undergo higher apoptosis compared to endothelial cells.

4.3 Prevalence of Vascular Smooth Muscle Cells

VSMCs are located throughout the large blood vessels. However, for isolation and culture of VSMCs, the following three large vessels are primarily used not only because of their large diameter and accessible locations but also because of their involvement in **atherosclerotic plaque formation.**

- Human ascending (thoracic) aorta
- Human descending (abdominal) aorta
- Pulmonary arteries

The most common sources for isolating VSMCs are the following:

- Aorta
- Coronary arteries
- Pulmonary arteries
- Other sources include the placenta, umbilical cord, and mammary blood vessels

4.4 Dissecting Instruments for Isolation of Smooth Muscle Cells

The instruments are described in the isolation and culture of vascular endothelial cells, Sect. 4 of this chapter.

4.5 Materials for Isolation and Culture of Vascular Smooth Muscle Cells

4.5.1 Sodium Pentobarbital

1 gm·ml^{-1} pentobarbital is used most often as the anesthetic for animals.

4.5.2 Phosphate-Buffered Saline

Sterile PBS without Ca^{+2} and Mg^{+2} is used to wash the sample tissue/organs.

4.5.3 70% Ethanol (ETOH)

ETOH is used as a disinfectant for the laminar flow hood working area and other materials.

4.5.4 Cell-Dissociating Enzymes

- Collagenase from *Clostridium histolyticum* stock (10 mg·ml^{-1}, freshly prepared).
- Add 10 mg collagenase in 1 ml HBSS, filter sterilize, and use immediately.
- Do not store an extra amount or reuse the latter. Final concentration needed is 0.5 mg·ml^{-1}.

4.5.5 Fungizone

0.25 mg·ml^{-1} fungizone is used as an antimycotic agent

4.5.6 Containers and Associated Materials

Flasks (25 mm/75 mm), plates (30 mm/60 mm/100 mm), (15 and 50) ml centrifuge tubes, and pipettes of various capacities are needed for cell culture. All these materials must be sterilized before use and should be disposable, of mammalian cell culture grade and polystyrene constituted. Membrane filters having various pore sizes are used for filter sterilization of the cell culture medium.

4.5.7 Cell Culture Medium of Vascular Smooth Muscle Cells

The complete medium is **DMEM** with 10% FBS, 2 mM L-glutamine, penicillin (100 U·ml^{-1}), and streptomycin (100 μg·ml^{-1}).

NB: Instead of using DMEM, many researchers use a medium comprising 1:1 DMEM and F12K.

4.6 Cell Division Capacity and Growth Properties of Vascular Smooth Muscle Cells

The VSMCs in the mature blood vessels rarely proliferate or migrate. However, these cells express various smooth muscle-specific contractile proteins such as α-SMA and calponin. Thus, these VSMCs contract as and when necessary.

On the contrary, whenever an injury happens to blood vessels, there is a significant phenotypic change in VSMCs, converting the contractile phenotype to proliferative and migrative phenotype. The extracellular matrix synthesis is also noticed under this condition.

- In 1993, *Kirschenlohr HL and colleagues* demonstrated that "VSMCs from adult human aortas proliferated in culture in response to FCS supplementation, with a population doubling time of **(70–85) hours** compared to **(35 ± 5) hours** for VSMC derived from adult rat aortas" (Kirschenlohr et al. 1993).
- In 2012, *Proudfoot D and colleagues* described that human aortic VSMCs have a typical doubling time of about **44 h** (Proudfoot and Shanahan 2012).

4.7 Isolation and Culture of Vascular Smooth Muscle Cells from Mouse Aorta

Several groups have established methods for isolating and culturing VSMCs from mouse aorta.

A discussion of some of these methods is as follows:

The method comprises the following steps:

- Use two ~10-week-old mice.
- Euthanize them by intraperitoneal injection of 0.25 ml ($1gm \cdot ml^{-1}$) sodium pentobarbital using a 26-gauge needle.

NB: Alternatively, one can asphyxiate mice by exposing them to CO_2 for 2 min.

- Spray mice with 70% ethanol.
- Place mice under a dissecting lamp with a magnifier.
- Surgically, open mice from the abdomen all along to the thorax one by one, without cutting any blood vessels to avoid bleeding.
- Collect the blood via cardiac puncture using a 1 ml syringe with a 26-gauge needle.
- Remove/discard all the organs except the heart to allow a clear view of the aorta.
- Place the mouse under a microscope.
- Dissect the aorta from its origin at the left ventricle to the iliac bifurcation.
- Leave the aorta attached to the left ventricle.
- Use a 3 ml syringe fitted with a 26-gauge needle to puncture the left ventricle.
- Perfuse with 3 ml sterile PBS, so that the aorta is flushed and all the blood cells are removed from the lumen.
- Remove the aorta, using sterile microdissecting scissors, and place in a 100 mm Petri dish containing a drop or two of fungizone solution.
- Remove fat from the aorta vicinity.
- When the vessel is free of the fibrous material and fat, make two cuts, one below the arch, and another in diaphragm proximity.
- Place the aortas into the PBS in a 15 ml conical flask and keep cool.
- Remove adventitia and endothelial layers.
- Remove aortas from the 15 ml conical flask.
- Rinse the aortas in PBS and put them in a dish afterward.
- Add proteolytic enzymes.
- Incubate the tray at 37 °C for 10 min.
- Put the aorta into a new culture plate and rinse with DMEM:F12 media (1:1) to wash off enzymes.
- Remove the medium and add a fresh enzyme solution and incubate at 37 °C in 5% CO_2 within the incubator for about an hour.
- Check whether the vessels are dissolved and the cells are floating.
- If not, the incubation time may be increased for another couple of minutes.
- Titrate the cells with a Pasteur pipette.
- Suspend and wash the cells with 4 ml of DMEM:F12 media (1:1), followed by centrifugation at 1500 RPM for 5 min.
- Decant the supernatant medium and wash the pellet once again by repeating the above step.
- Remove the supernatant medium.
- Suspend the cells in 1.5 ml DMEM: F12 media (1:1).
- Put the cell suspension in 3 wells (0.5 ml each) of 48-well cell culture dish.

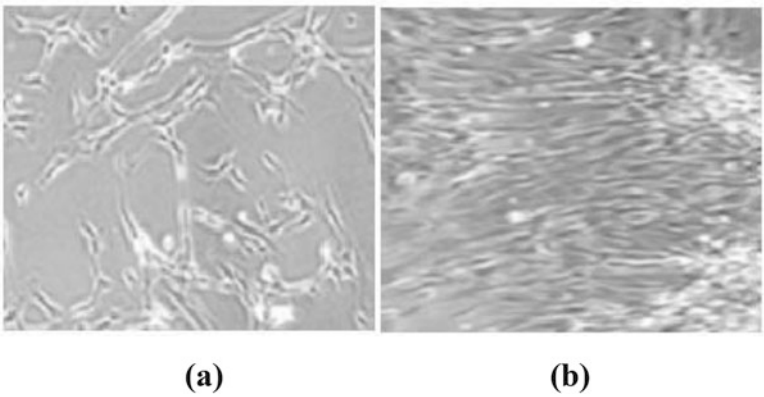

Fig. 6 Growth pattern of VSMCs: (a) hill and (b) valley morphologies

- Grow for 1 week, till 80–90% confluency.
- Subculture the cells and grow for 3–5 passages.

4.7.1 Growth Pattern of the Isolated Cells.

VSMCs' growth characteristics exhibit hill and valley patterns **(**Fig. 6**)**.

4.7.2 Confirmatory Identification of Vascular Smooth Muscle Cells

- VSMCs can be confirmed by positive immunohistological staining for α-smooth muscle actin, using a commercial kit and hematoxylin-assisted counterstaining.
- Besides α-smooth muscle actin, CD3 (lymphocytes), CD68 (macrophages), and CD31 (endothelial cells) are also recognized as negative VSMC screening markers.

4.7.3 Passaging Vascular Smooth Muscle Cells

- Split the cells into 48-well plates as follows:
- 1–3 wells (1^{ST}p) up to 6 wells (2ND p) to 12 well (3RD p) to 24 wells (4TH p) to 60 wells (p).

Do not grow SMCs for more than (10–12) passages because of the following reasons:

- Changes in cell function
- Loss of growth capacity
- Spreading of cells

4.8 Isolation and Culture of Vascular Smooth Muscle Cells from Human Aorta

In 2012, Proudfoot and Shanahan established a detailed method for isolating and culturing VSMCs from human blood vessels (Proudfoot and Shanahan 2012)**.**

A detailed and complete protocol is available from their publication.

4.9 Identification of Vascular Smooth Muscle Cells Based on Phenotype (Contractile vs. Synthetic Phenotype)

4.9.1 Diameter of Vascular Smooth Muscle Cells

The largest SMCs exist in the uterus during pregnancy **(12 × 600) μm.**

The smallest are found in small arterioles **(1 × 10) μm.**

4.9.2 Shape of Vascular Smooth Muscle Cells Based on Their Phenotype

- Smooth muscle consists of spindle-shaped cells. However, unlike some terminally differentiated cells, VSMCs maintain marked phenotypic plasticity.
- VSMCs exhibit two distinct phenotypes, **contractile** and **synthetic**.

A brief discussion of phenotypes is as follows:

4.9.3 Contractile Phenotype of Vascular Smooth Muscle Cells

- Under normal physiological conditions, VSMCs remain in a contractile phenotype. This phenotype is only present in intact blood vessels or very early primary cultures.

The contractile phenotype of VSMCs is characterized by the following:

- High expression of contractile proteins (e.g., α-SMA).
- Low in the rough endoplasmic reticulum (rough ER).
- Small Golgi apparatus.
- Low proliferative index and fusiform morphology (Fig.7).
- This phenotype is also called "differentiated phenotype," but not "terminally differentiated phenotype" (Beamish et al. 2010).

4.9.4 Synthetic Phenotype of Vascular Smooth Muscle Cells

- Within a few days of 2D culture, VSMCs transform from a contractile to synthetic phenotype, also known as ***"de-differentiated phenotype."***

This dedifferentiated VSMCs' phenotype is characterized by the following:

- Gradual loss of myofilament bundles.
- Formation of an extensive rough endoplasmic reticulum (rough ER) (Fig.8).

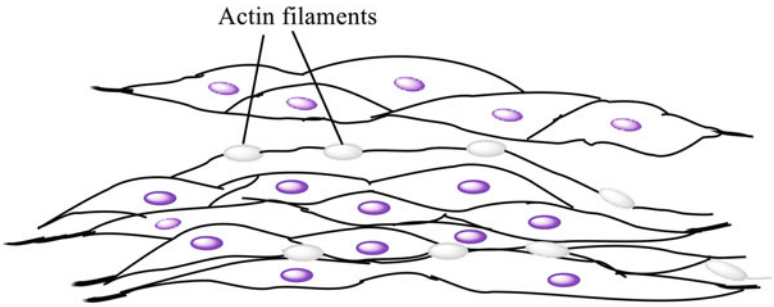

Fig. 7 Contractile phenotype of SMCs has actin expression as their hallmark

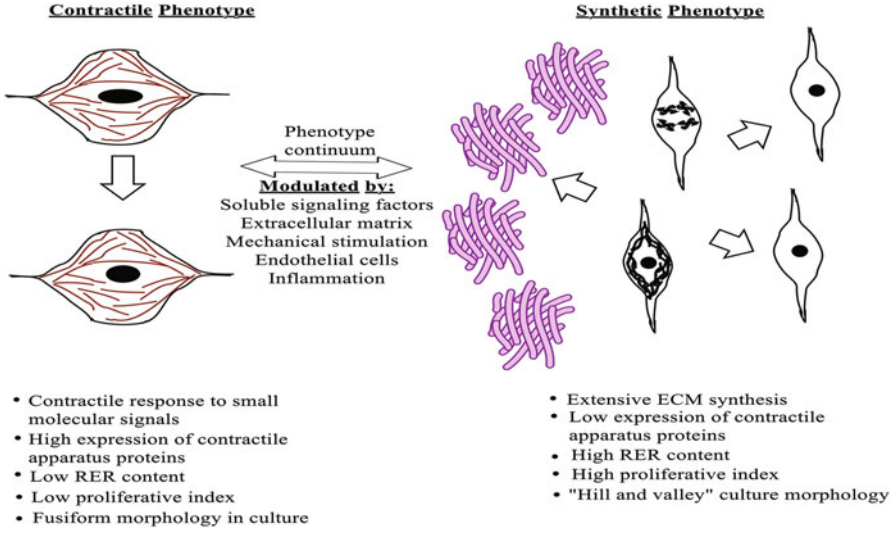

Fig. 8 Distinction of contractile and synthetic VSMC phenotypes

- Large Golgi complex.
- This evolution provides VSMCs with the ability to synthesize DNA and divide when stimulated with serum or purified growth factors (Pahk et al. 2017).

NB: Recent studies indicate that some SMCs exhibit a synthetic phenotype even under* in vivo *conditions.

In a significant 2010 study, ***Beamish JA and colleagues*** described the molecular regulation of contractile smooth muscle cell phenotype and its implications for vascular tissue engineering (Beamish et al. 2010).

4.10 Identification of Vascular Smooth Muscle Cells Based on Marker Proteins Expression

4.10.1 Markers for Contractile Phenotype of Vascular Smooth Muscle Cells

The best markers for the contractile phenotype in VSMCs are tropoelastin, a matrix protein; α-smooth muscle actin, γ-smooth muscle actin, calponin, and phospholamban.

In 1993, *CM Shanahan and colleagues* identified SM22α as VSMCs' contractile protein. They also identified CHIP28, a putative membrane channel protein that is not highly expressed in other SMCs as a new VSMC marker.

The developing bone marker osteopontin and matrix Gla protein (MGP) are strongly expressed in aortic VSMCs, but not in other SMCs. These proteins are responsible for calcification that commonly occurs in vascular lesions.

Recent studies indicate that smooth muscle myosin heavy chain (SM-MHC) and smothelin are specific markers *for a contractile phenotype.*

4.10.2 Marker Proteins for Synthetic Phenotype of Vascular Smooth Muscle Cells

- Identifying features of an SMC synthetic phenotype include elevated collagen type III, cyclophilin A and matrix metallopeptidase-9 in the neointima, elevated FDG uptake into the atherosclerotic carotid artery, and acutely high glucose transporter 1 expression in the neointima.

4.11 Contractile to Synthetic Phenotype Transformation of Vascular Smooth Muscle Cells

1. Vascular smooth muscle cells (VSMCs) are capable of both contractile and synthetic functions, although, in native form, they exist primarily in contractile phenotypes.
2. Three major factors have been identified that enable the transition of the contractile phenotype to the synthetic form. These are an expression of specific biochemical factors, ECM components, and physical factors including stretch and shear stress.
3. Studies reveal a decrease in contractile marker protein expression levels upon culturing SMCs. Slowly, the expression intensity of contractile proteins decreases while that for synthetic proteins increases.
4. Biochemical factors influencing the SMC phenotype include PDGF, transforming growth factor-beta (TGF-β), activin a, retinoid, angiotensin II, and tumor necrosis factor-α (TNFα).
5. For example, the two PDGF isoforms, namely, PDGF-A and PDGF-B, induce a more synthetic phenotype in human adult SMCs. PDGF-B also induces a contractile pig coronary artery to develop a rhomboid synthetic morphology in an elevated proliferation.

6. In contrast to PDGF, the TGF-β isoforms are deemed essential for contractile SMC phenotype formation.
7. Other ECM constituents that foster a contractile SMC phenotype include fibrillar collagen type I, collagen type IV, and laminin, all preferentially favoring the contractile phenotype.
8. While most ECM proteins support a contractile phenotype, fibronectin preferentially supports a synthetic phenotype.
9. Hyaluronan, a major ECM glycosaminoglycan, contributes to atherosclerosis progression and also favors the synthetic phenotype.
10. The specific organization of ECM proteins also affects the VSMCs' phenotype. As one example, 3D cultures enable higher collagen I expression and consequently favor a synthetic phenotype.
11. Thus, both the composition and organization of ECM components influence SMC phenotype (Rensen et al. 2007).

4.12 The Rationale for Culturing Vascular Smooth Muscle Cells

- VSMCs create the tunica media of mammalian arteries and regulate vasomotion/vascular tone by contraction and relaxation.
- VSMCs in culture serve as an important aid to investigate the mechanism by which VSMCs contribute to vessel wall contraction.
- Since VSMCs are one of the important constituents of major blood vessels, they perform a decisive role in vasculogenesis and angiogenesis, restenosis, and thrombosis.
- Like all other cells, VSMCs are also generated from the preexisting VSMCs or progenitor VSMCs by the process of proliferation and pericyte maturation.
- Experimentally, it is observed that alteration of a specific VSMC phenotype may be related to one or more specific pathophysiological conditions.

5 Isolation and Primary Culture of Vascular and Nonvascular Fibroblasts

5.1 Properties of Fibroblasts

- Fibroblasts are a type of connective tissue cells of mesenchymal (mesodermal) origin that produce collagen, elastin, and various other ECM components.
- In the large blood vessels, fibroblasts are present in the tunica adventitia (the outer layer).
- In certain conditions, epithelial cells can give rise to fibroblasts, through epithelial–mesenchymal transition (EMT).
- On the other hand, in the process of development, tissue repair, and tumor/cancer growth, fibroblasts may undergo mesenchymal–epithelial transition (MET).

5.1.1 The Relationship Between Fibroblasts and Fibrocytes

Fibroblasts and fibrocytes are two states of the same cells. The less active state of fibroblasts is called fibrocytes, typically responsible for maintenance and tissue metabolism.

5.2 Locations of Fibroblasts in Humans

5.2.1 Blood Vessels

Tunica adventitia, comprising the outer layer of macrovessels containing fibroblasts.

5.2.2 Heart

The heart contains special fibroblasts, called **myofibroblasts.**

5.2.3 Skin

The dermis layer of the skin contains fibroblasts.

5.2.4 Lung

The airways and interstitium of the lung contain fibroblasts.

5.2.5 Alimentary Canal

The matrix of the digestive tract contains fibroblasts.

5.2.6 Reproductive System

The vas deference and uterus (stroma) contain fibroblasts.

NB: Myofibroblasts are the cells representing an intermediate phenotype between fibroblasts and smooth muscle cells.

5.3 Dissecting Instruments for Isolation of Tissues and Organs Containing Fibroblasts

The required instruments are described in the isolation and culture of vascular endothelial cells, Sect. 4 of this chapter.

5.4 Materials for Isolation and Culture of Fibroblasts

The following materials are needed for fibroblast isolation and culture:

5.4.1 Sodium Pentobarbital

- 1 gm·ml^{-1} of pentobarbital is used as an anesthetic for animals.
- Use directly from the stock without any dilution.

5.4.2 Dulbecco's Phosphate-Buffered Saline

Sterile Dulbecco's phosphate-buffered saline (DPBS) is used to wash the tissue/organ samples.

5.4.3 Hanks' Balanced Salt Solution

Hanks' balanced salt solution (HBSS) is used for washing buffer and digestive solution.

5.4.4 70% Ethanol

70% ethanol is used as a disinfectant for the laminar flow hood working area and other materials.

5.4.5 Cell-Dissociating Enzymes

- Collagenase from *Clostridium histolyticum* stock (10 $mg \cdot ml^{-1}$ freshly prepared).
- Add 10 mg collagenase in 1 ml HBSS, filter sterilize, and use immediately.
- Do not store or reuse it later.
- The final concentration is 0.5 $mg \cdot ml^{-1}$.

5.4.6 Penicillin/Streptomycin

- Use as 1% (v/v) from the stock.
- The final concentrations of penicillin and streptomycin are (100 $U \cdot ml^{-1}$) and (100 $\mu g \cdot ml^{-1}$), respectively.

5.4.7 Fungizone

- 0.25 $mg \cdot ml^{-1}$ fungizone is used as an antimycotic agent.
- Use a final concentration of (0.25–2.5) $\mu g \cdot ml^{-1}$.
- Nystatin dehydrate can be used instead of fungizone.

5.4.8 DNase I from Bovine Pancreas

- Dilute 10 mg DNase I into 10 ml HBSS.
- Filter-sterilize aliquots and store at -20 °C.
- Use 100 $\mu g \cdot ml^{-1}$ as the final concentration.

5.4.9 0.1% Gelatin

Mammalian cell culture containers must be coated with 0.1% sterile gelatin as described in endothelial cell culture, a cell-adhesive agent in the culture containers.

5.4.10 Mammalian Cell Culture Containers and Associated Materials

- Flasks (25 mm/75 mm) or plates (30 mm/60 mm/100 mm), (15 and 50) ml centrifuge tubes, and pipettes of various capacities are needed for cell culture.
- All these materials are sterile, disposable, typically of mammalian cell culture grade, and made up of polystyrene.
- Membrane filters having various pore sizes are used for filter sterilizing the cell culture medium.

5.4.11 Cell Culture Medium for Fibroblasts Culture

- The complete medium contains DMEM with 4.5 g D-glucose, 10% FBS, 1% L-glutamine (2 mM final), 4 ng·ml^{-1} fibroblast growth factor (FGF), penicillin (100 U·ml^{-1}), and streptomycin (100 μg·ml^{-1}).
- If necessary, sodium bicarbonate may be added to adjust the medium pH.

The following are examples of methods for isolating and culturing fibroblasts:

5.5 Isolation and Culture of Fibroblasts from Mouse Lung Using Enzymatic Digestion

- Fibroblasts are fast-growing and can be rapidly expanded from small samples.

Fibroblasts can be isolated by any of the following three procedures:

- Enzymatic digestion (e.g., with collagenase or trypsin) of the tissue pieces.
- Explant culture of the small-sized ((1–3) mm in diameter) tissue pieces.
- A combination of enzymatic digestion and explant culture.

NB: Some protocols recommend that fibroblasts be cultured in a 3% (rather than 20%) oxygen environment.

- Some protocols recommend the use of fetal serum protein fetuin in the cell culture medium for more robust growth.
- Important publications in the field of fibroblast culture are credited to Vangipuram et al. 2013; SeluanovA and Gorbunova 2010; Keira et al. 2004; and Takashima 2001.

5.5.1 Isolation and Culture of Lung Fibroblasts

This protocol is a modified version of Das et al. 2002**.**

- Take three mice, each at least 10 weeks old.
- Euthanize the mice by intraperitoneal, 0.25 ml sodium pentobarbital injection using a 26-gauge needle.
- Alternatively, asphyxiate mice with CO_2 for 2 min.
- Spray the mice with 70% ethanol.
- Place the mice under a dissecting lamp with a magnifier.
- Surgically open all the mice (from the abdomen to the thoracic region) without cutting any blood vessels to avoid bleeding.
- Now expose the lungs and perfuse them with sterile tissue culture grade DPBS, and remove the system lungs–trachea–heart from the mice body.
- Discard the heart and trachea.
- Make 15 ml washing solution by adding penicillin (100 U·ml^{-1}) and streptomycin (100 μg·ml^{-1}) final concentration in HBSS.
- In a Petri dish, wash the lungs with the washing solution and place them in a Falcon tube in the washing solution.

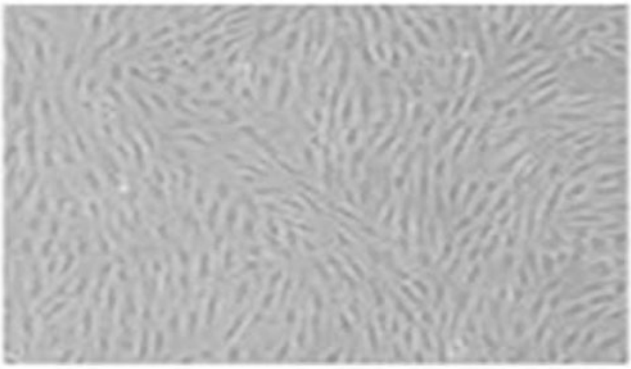

Fig. 9 Typical morphology of mice fibroblasts, segregated from pulmonary artery adventitia using the limited dilution cloning method. As visible, the isolated cells revealed a **rhombus morphology**. The study referred to here also isolated the fibroblasts from adventitial fibroblasts, which revealed a spindle shape (not shown here). Together, the differential origin fibroblasts were characterized by round or elongated morphology. (Das et al. 2002)

- Close Falcon tubes inside a lab hood and transfer the Falcon tubes to the cell culture hood.
- Open the tubes inside the hood.
- In a Petri dish, cut the lungs of the three mice into small pieces (2 mm in diameter) using a previously unused scalpel blade.
- Collect the lung pieces in one big Falcon tube.
- Make 15 ml digestion solution by adding 0.75 ml collagenase stock, 1.5 ml DNase stock, 150 μl penicillin/streptomycin stock, and then making to 15 ml using HBSS.
- Add 1.5 ml digestion solution to the Falcon tube containing the lung pieces and incubate inside a shaking water bath at 37 °C.
- After 10 min, pipette the solution inside the culture hood and dilute to neutralize proteolytic enzymes.
- Repeat the extraction six times (10 min $\times$ 6 = 60 min) to get a sufficient number of cells.
- Combine the extracts and centrifuge at 1200 rpm, 4 °C for 10 min to remove collagenase.
- Resuspend the cells in a 10 ml complete medium and place them in a T-75 flask.
- Place the flask in the CO_2 incubator.
- While live fibroblasts adhere to the plates, the dead cells float.
- Replace the medium the following day.
- If cells seem too sparse, replace the medium after 3 days instead of 1 day.
- While changing the medium, the floating dead cells will be removed.
- Subculture the cells as described for other mammalian cells.
- Figure 9 depicts the typical morphology of mice fibroblasts isolated from pulmonary artery using the limited dilution cloning method, wherein a rhombus morphology is inferred.

NB: Mouse fibroblasts are sensitive to oxidative stress and will senesce within ~ 14 population doubling times when maintained at 20% (atmospheric) oxygen.

Mouse fibroblasts can be maintained for more passages when maintained at 3% O_2.

5.6 Isolation and Culture of Fibroblasts from Rat Coronary Artery

In 2007, Jenkins and associates established a rat coronary artery fibroblast cell culture model.

- They used tunica adventitia of the artery to isolate fibroblasts.
- For detailed protocol, refer to ***Jenkins* et al.** 2007 publication *(*Jenkins et al. 2007*)*.

5.7 Isolation and Culture of Fibroblasts from Human Skin

- Fibroblasts are one of the important constituent cells of dermal (skin) tissues. The culture of skin fibroblasts would help to understand the skin connective tissue production, as well as the characteristic skin pathophysiology.
- In vitro cultured primary skin fibroblasts are widely utilized to understand the process of wound healing.
- Other major experiments that are conducted on in vitro cultured skin fibroblasts include studying the effects of various growth factors, toxicity studies, various drugs, and others.

NB: For culturing skin fibroblasts, the epidermis is either dissected out or enzymatically removed to prevent epidermal cell contamination into the skin fibroblasts culture.

The presented protocol is a modification of the original protocol, standardized by Keira et al. 2004**.**

This is an explant culture method, generally accepted as the best one.

5.7.1 Initial Processing of Human Skin Specimen

- For various reasons, portions of the human subjects (patients), that is, skin are required to be dissected or surgically removed by specialized doctors.
- After receiving written consent from the subject concerned, the skin sample is collected in a 50 ml sterile tube containing DMEM.
- The sample can be processed immediately in a laminar flow hood or stored at 4 °C, if it needs time but must be processed within 4 h of collection.
- It is recommended that the collected skin sample should be cleaned multiple times (exhaustively) with sterile PBS before processing.

Fig. 10 Skin fragments in the cell culture medium for attachment and growth

5.7.2 Primary Culture of Human Dermal Fibroblasts

- As mentioned above, the original protocol has been borrowed from the work of Keira et al. 2004.
- This is an **explant culture** method.
- Experimentally, the epidermis is removed from the dermis after which the isolated dermis is fragmented into 03 mm pieces.
- Now, 60 mm Petri dishes are taken and 0.5 ml cell culture medium is added. Henceforth, the dermis fragments are added to the culture plates.
- The Petri dishes are maintained in a semi-open state in a laminar flow for 40 min to adhere to the dermis specimen along the culture surface **(**Fig.10**)**.
- After 40 min of incubation, 5 ml of DMEM with 20% FBS, penicillin (100 $U \cdot ml^{-1}$), and streptomycin (100 $\mu g \cdot ml^{-1}$) are added to the culture plates. Now the culture plates are incubated at 37 °C in a CO_2 incubator under a 5% CO_2 environment.
- Change the medium at every 2 days interval.
- Approximately in 1-week time, fibroblast proliferation is observed.

5.8 Identification of Fibroblasts Based on Their Phenotype

5.8.1 Diameter of Fibroblasts

(10–15) μm.

5.8.2 Shape of Fibroblasts

Fibroblasts can take a **wide array of shapes in different tissues**.

Typically, fibroblasts acquire either bipolar or multipolar morphology.

5.8.3 Phenotype of Fibroblasts

- Fibroblasts are large, flat, elongated (spindle-shaped), or round-shaped with extensions radiating out from the ends of the cell body.
- The cell nucleus is flat and oval.

5.9 Identification of Fibroblasts Using Marker Proteins Expression

- Fibroblasts are identified based on their spindle shape combined with positive staining for the mesenchymal (mesodermal) marker vimentin.
- By immunohistochemistry (IHC), vimentin and TE-7 identify both dermal and lung fibroblasts.
- The fibroblast surface antigen (**FSA**), a glycoprotein produced by connective tissue cells (mesenchymal cells, fibroblasts, and astroglial cells), can also be used for fibroblasts and fibroblast cell line detection.
- Fibroblasts present in the skin, alveolus, and blood vessels express CD34, a marker of hematopoietic stem cells (HSCs).
- Other markers of fibroblast differentiation in the human dermis are endogenous peroxidases.
- By immunohistochemistry, heat shock protein 47 (HSP47) can also be used as a marker for skin fibroblasts (Kuroda and Tajima 2004).

5.9.1 Significance of Fibroblasts Culturing

- Fibroblasts do not carry mutations in their proto-oncogenes and tumor suppressor genes. Therefore, these types of cells possess a normal cell cycle, rather than defected one, as observed in tumor and cancer cells. These cells are used to understand the normal cell cycle, cell synchronization, and its regulation by various molecules, and finally differences in cell cycle between a normal cell and a tumor or cancer cell.
- Fibroblasts are an important example of cells to understand the normal process of cell proliferation by the process of mitosis, the normal process of programmed cell death or apoptosis, and the process of various kinds of DNA repair in completely normal cells.
- The fibroblasts of the blood vessels produce collagen that provides structural support by anchoring the blood vessels to nearby tissues.
- Fibroblasts also play a critical role in wound healing.
- Abnormal proliferation, activation, or differentiation of fibroblasts to myofibroblasts causes excessive synthesis and accumulation of collagen (a connective tissue material) and other ECM materials, leading to tissue fibrosis.
- As discussed previously, in the vascular wall the fibroblasts are present in the tunica adventitia. During the process of vascular injury, these cells get activated, proliferate, and respond to establish normalcy.
- The adventitial fibroblasts produce cytokines and chemokines during pathological conditions. In the next step, these cytokines and chemokines induce

infiltration of immunological cells into the adventitial layer of a vessel wall. Immune cell infiltration into the adventitia results in adventitial inflammation and can lead to cardiovascular diseases (CVDs).
- Fibroblasts are suitable for functional, biochemical, and genomic studies.
- Collectively, all the above observations indicate that fibroblasts provide an ideal cell model system for studying various normal physiological events and diseased states (An et al. 2015).

5.9.2 Examples of Studies One Can Conduct Using Fibroblasts

The significance of the fibroblast culture section has discussed the importance of fibroblast culture.

Briefly, the fibroblast culture has the following importance:

- Understanding the normal cell cycle, cell proliferation, and differentiation.
- Understanding the differences between normal cells and cancer cells, particularly concerning cell cycle regulation.
- Screening for drugs and toxins.
- Gene delivery to cells.
- Genome editing or CRISPR technology application.
- Understanding the process of wound healing.
- Understanding the physiology and various pathophysiologies of skin.

6 Isolation and Primary Culture of Epithelial Cells

6.1 Properties of Epithelium

The epithelium is the thin tissue layer, either single or multiple, forming the inner or outer surface of organs, glands, the mouth, nostrils, trachea, alveoli, ducts lumen of mammary glands, the lining of the alimentary canal, and urinary bladder.

Salient features of epithelium include the following:

- All surfaces, including **cutaneous, serous, and mucus,** are covered by epithelium.
- **No blood vessels** are present in the epithelium.
- The epithelium is not present in the inner lining of blood or lymph vessels. Endothelial cells replace the epithelial cells/epithelium in these vessels.
- Epithelial cells form the epithelium.

6.2 Differences Between Endothelial and Epithelial Cells

- Endothelial cells are present in the endothelium, which is the innermost layer of the blood vessels or circulatory vessels. These cells are never exposed to exteriors. The epithelial cells are present in the epithelium, which cover the structures

exposed to the exterior surfaces of the body (e.g., skin, intestine, urinary bladder, urethra, and many other organs).

- The endothelium is made up of a single layer of endothelial cells. On the other hand, the number of layers for epithelial cells (in an epithelium cell) varies in different tissue layers.
- Both endothelial and epithelial cells are of epithelial origin. However, while endothelial cells have **vimentin,** epithelial cells have **keratin.**
- Endothelial cells also contain von Willebrand factor or factor 8 antigen. Epithelial cells do not possess the von Willebrand factor.
- Endothelium provides a **non-thrombogenic surface, which is not so for e**pithelium.

6.3 Differences Between Epithelial Cells and Fibroblasts

- Epithelial cells are tightly connected and arranged in monolayers. They have numerous functions, including protection, diffusion, secretion, absorption, excretion, and separation of compartments. In contrast, fibroblasts comprise the structural framework of tissues and synthesize ECM.
- Epithelial cells undergo growth arrest in response to TGF-β, whereas fibroblasts undergo morphological changes and proliferate in response to TGF-β.
- Fibroblasts have much higher 5′-nucleotidase activity than epithelial cells and exhibit a faster 8-azaguanosine-5′-monophosphate degradation to 8-azaguanosine. As a result, epithelial cells (isolated from rat liver) are more sensitive to the toxic effects of the purine analog, 8-azaguanine.
- Fibroblasts can migrate as individual cells while epithelial cells cannot.
- Epithelial cells express the markers, pan-cytokeratin, CK8, and E-cadherin while fibroblasts express a high α-SMA.

6.4 Foremost Locations of Epithelial Cells in Humans

- Striated skin layers.
- The lining of the mouth, taste buds, nose, trachea, alveoli, alimentary canal, including esophagus, stomach, small and large intestine, urinary and gall bladder.
- Organelles in the kidney, pancreas, ear, and eye.
- Ducts and glands such as the bile ducts and salivary gland.
- Male and female primary and secondary reproductive organs include the testes, prostate, ovary, uterus, and mammary glands.

6.5 Classification of Epithelium Based on the Cell Layering

6.5.1 Simple Epithelium

The epithelium is composed of a single layer of cells.

6.5.2 Stratified Epithelium

This epithelium comprises multiple layers of cells.

6.5.3 Pseudo-Stratified

Epithelium of this regime possesses fine hair-like extensions, cilia, unicellular glands, and goblet cells that secrete mucus.

6.6 Additional Classifications of Epithelium

6.6.1 Keratinized Epithelium

- The most exterior or apical layer of dead cells contains keratin as a resistant protein.
- An example of this type of epithelium is found in mammalian skin, conferring water-resistant ability.

6.6.2 Transitional Epithelium

- This type of epithelia is found in tissues such as the urinary bladder, wherein cells change their shape due to stretching.
- Here, columnar epithelium may be converted to the cuboidal epithelium.

6.7 Dissecting Instruments for Isolating Epithelial Cells

The instruments are described in the isolation and culture of vascular endothelial cells, Sect. 4 of this chapter.

6.8 Materials for Isolation and Culture of Epithelial Cells

- This section describes the isolation and culture of epithelial cells from the trachea and alveoli of lung and mammary glands.
- The materials for isolation and culture of these cells are mentioned in their specific section on isolation and culture.

6.9 Isolation and Culture of Pulmonary Tracheal Epithelial Cells

Human pulmonary epithelial cells can be isolated either from the trachea or bronchioles or from the alveolus. In general, the trachea and bronchi are lined by a pseudo-stratified mucociliary epithelium, consisting of three cell types, as follows.

6.9.1 Basal Cells

Around 30% of tracheal epithelial cells are basal cells. These cells serve as a pool of progenitor cells that can repopulate a damaged epithelial cell layer.

6.9.2 Ciliated Cells

These cells propel mucus and clear particles from the respiratory tract.

6.9.3 Secretory Cells

These cells secrete mucus and other factors contained within the mucus layer.

Pulmonary epithelial cells can be cultured in two distinct ways:

Air Interface Culture

With this method, airway epithelial cells can be grown on porous membranes with the medium in the basolateral chamber but not on the apical side. The primary importance of this culture is to mimic the air-exposed environments to have similar in vivo airway conditions.

Liquid Submerged Culture

In this method, cells can be cultured with the medium on both sides, meaning cells are submerged in the medium.

Here, we discuss first the isolation and culture of tracheal and then alveolar, epithelial cells.

6.10 Comments Regarding Isolation and Culture of Pulmonary Tracheal Epithelial Cells

- It is very difficult to get trachea or human lung samples except during lung transplantation or accidental death.
- Additionally, important ethical issues limit the collection of human organs and tissues.
- Therefore, animals such as mice and rats are predominantly utilized to isolate tracheal epithelial cells.
- There are several protocols for isolation and culturing respiratory epithelial cells.

Some landmark studies in epithelial cell culture include the following:

- In 1977, ***Collier*** **et al.** described tracheal ring organ cultures**.**
- In Goldman and Baseman 1980, ***Goldman*** **et al.** described the enzymatic dissociation of epithelial cells followed by an investigation of various culture conditions.
- In 1988, ***Whitcutt*** **et al.** described the air–liquid interface (ALI) for the culture of tracheal epithelial cells.

6.11 Isolation and Culture of Human Tracheal Epithelial Cells

The method described here is a minor modified version of the one demonstrated by Bals **et al.** ***in*** 2004 ***with a minor modification.***

- It involves ALI cultures of human airway epithelial cells.
- There are two well-standardized protocols. The first one involves the culture of human epithelial cells from large airways while the second protocol involves the culture of human epithelial cells from distal airways. In both methods, cells are obtained from human lung pieces collected during surgery. The following sections describe the steps involved in human tracheal epithelial cell isolation and culture.

6.12 Reagent Preparation for Isolation and Culture of Human Tracheal Epithelial Cells

6.12.1 Preparation of Incubation Medium for Isolating Epithelial Cells from Large Airways

Dulbecco's modified eagle medium (DMEM) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), tobramycin (40 μg/ml), ceftazidime (50 μg/ml), amphotericin B (2.5 μg/ml), imipenem-cilastatin (50 μg/ml), DNase (10 μg/ml), and dithiothreitol (0.5 mg/ml).

NB: All chemicals can be obtained from Sigma.

6.12.2 Preparation of Incubation Medium for Isolating Epithelial Cells from Distal Airways

DMEM/Ham's F12 medium supplemented with penicillin (100 U/ml)/streptomycin (100 μg/ml) gentamicin (0.5 mg/ml), and amphotericin B (10 μg/ml). For cystic fibrosis lung specimens, add ceftazidime (500 μg/ml) and ticarcilline (500 μg/ml).

6.12.3 Digestion Medium for Isolating Epithelial Cells from Large Airways

Incubation medium +0.1% protease 14 (Sigma).

6.12.4 Digestion Medium for Isolating Epithelial Cells from Distal Airways

MEM/Ham's F12 medium supplemented with 0.1% protease and 0.1% DNase.

6.12.5 Coating the Cell Culture Containers for Large Airways with Adhesive Agents

- Transwells are coated with collagen type 1.
- To prepare collagen, add 1 mg collagen to 1 ml 0.1 M acetic acid. Stir at room temperature for (1–3) hours or until collagen dissolves.
- Dilute ten-fold in distilled water to a resulting 0.1% working solution, store at 4 °C, and use within 4 weeks.
- To coat 1.2 cm inserts, add 100 μl collagen, whereas for 3 cm inserts, add 1 ml collagen.
- Incubate the collagen-filled culture vessels overnight at 37 °C.

- The next day, remove excess fluid from the coated culture containers and dry the culture containers for 30–60 min.
- The coated flasks are sterilized via exposure to UV light for 2–3 h in a laminar flow hood.

6.13 Coating the Cell Culture Containers for Distal Airways with Adhesive Agents

For distal airways, use a homemade collagen membrane affixed to Plexiglas support.

6.13.1 Preparation of Medium for Air–Liquid Interface Cell Culture from Large Airways

As discussed in the previous section, this protocol is adapted from the research work of Bals et al. 2004 ***with minor modifications.***

- Ultroser G2 is supplemented to the DMEM/Ham's F12.
- Mostly, the large airways, trachea, or main bronchus are dissected.
- Start the proximal dissection and proceed distally.
- Airways: DMEM/Ham's F12 medium supplemented with insulin (5 μg/ml), transferrin (7.5 μg/ml), hydrocortisone (10^6 M), endothelial cell growth supplement (2 μg/ml), EGF (25 ng/ml), triiodothyronine (3×10^8 M), L-glutamine (1 mM), penicillin/streptomycin (100 μg/ml), gentamicin (50 μg/ml), and amphotericin B (5 μg/ml).
- For cystic fibrosis cultures, add ceftazidime (125 μg/ml) and tobramycin (100 μg/ml).
- Chemicals should be procured from Sigma/other standard companies.

6.14 Isolation and Culture of Epithelial Cells from Large Airways

As discussed in the previous section, this protocol is adapted from the research work of Bals et al. 2004 ***with minor modifications.***

- Retrieve resected lung tissue prepared on a clean working surface and store in sterile PBS without Ca^{+2} and Mg^{+2} for up to 2 h (for laboratory transport of a sample).
- Before processing, aseptically clean the large airways multiple times in sterile PBS.
- The attached soft tissue or lung parenchyma is dissected.
- Open the airways longitudinally by cutting.
- Incubate in an incubation medium for 6–24 h at 4 °C.
- Remove medium and replace it with the digestive medium before storing at 4 °C for 2 h.
- Now, transfer the digestion solution and the airway into plastic dishes.

- Hold the airway with forceps and use a scalpel to scrape the luminal surface of the airway 4–5 times.
- The luminal epithelial cells detach and gradually come into the solution.
- The rest of the airway material is discarded and put into a biohazard disposal container.
- Spin down the cell suspension at 170 × *g* for 10 min.
- Remove the supernatant and resuspend the cell pellet in the **bronchial epithelial cell growth medium (BEGM).**
- To remove mucus and other materials present in the cells, spin the solution at 40 × *g* for 30 s.
- The epithelial cells containing supernatant are transferred into a culture vessel (size depends on the number of cells) and cultivate at 37 °C in a humidified atmosphere of 5% CO_2 air for 24 h.
- Following incubation, spin down the cells using low-speed centrifugation.
- Now that the cells are ready for being seeded into the transwells coated with collagen (collagen type I, Sigma C9791), wet the membrane using a 10-min immersion in the medium before emptying the wells.
- Fill the lower reservoir first with culture medium (1 ml for large 3 cm inserts, 0.2 ml for smaller 1.2 cm inserts).
- Fill the upper reservoir with a culture medium containing the appropriate number of cells to obtain a density of approximately 1×10^6 cells/cm^2 (0.5 million cells for small inserts and 4×10^6 cells for large inserts).

NB: The lower reservoir should be guarded for possible contamination with the cell-containing solution.

6.15 Isolation and Culture of Epithelial Cells from Distal Airways

As discussed in the previous section, this protocol is adapted from the research work of Bals et al. 2004 ***with minor modifications.***

- Collect the lung pieces.
- Wash with the incubation medium.
- Place in a clean location for further processing.
- Identify bronchioles by the devoid wall cartilage and ~ 1 mm outer diameter.
- Now remove the larger structures such as bronchi and vessels.
- Remove lung parenchyma from bronchioles using sharp curved scissors and binoculars.
- Cut the bronchioles into small segments (3–10 mm).
- Put the bronchiolar segments into a dish containing the incubation medium at 4 °C.
- For cystic fibrosis (CF) bronchioles, put the segments for at least 4 h in the medium to purge the contaminated bacteria (if any).

- Cut and open the bronchioles longitudinally to expose the epithelium to the digestion medium.
- Put the containers at 4 °C for incubating the bronchioles in the digestive medium.
- The epithelial cells would be gradually detached, coming into the digestive medium.
- Following incubation, neutralize the enzymes by adding 10% FBS to the digestion medium.
- Now remove the undigested bronchioles.
- Perform low-speed centrifugation of the epithelial cells.
- Resuspend the pellet into the culture medium.
- Observe the cells in a microscope and count their number.
- Fill the lower reservoir with the culture medium.
- Seed the cells on the collagen membrane (**Invitrogen**) at a **4×10^4 cells/mm^2** loading rate.

6.16 Culture After Seeding

- Following seeding, incubate the culture containers in a CO_2 incubator at 37 °C in a 5% CO_2, 95% moisture, environment.
- Cells should be settled and may be confluent if checked within 24 h of seeding.
- For **bronchiolar** but not bronchial cultures, no medium is added to the cell surface after seeding.
- For bronchial cultures, remove the apical medium when cells are completely confluent, (usually after 3 days).
- Subsequently, PBS is used to wash the apical side of the epithelial layer for 2–4 days.
- Every 24 h, the basolateral culture medium would be changed.

NB: Avoid contaminating the upper reservoir with the basolateral culture medium.

- At this juncture, the cell differentiation could be monitored using microscopical inspection of beating **cilia** and using an **ohmmeter** to measure the transepithelial electrical resistance.

6.17 Isolation and Culture of Porcine Tracheal Epithelial Cells

***This method was established by Yu W,* et al *with a little modification*. *Here are the step-by-step procedures established by the scientists*.**

The authors claimed that every material used in this procedure, including the small instruments, should be sterile besides doing all the isolation work inside the laminar flow hood.

- Remove the skin with the help of clean surgical scissors and scalpel.
- Cut along the sternum to open the upper abdomen.
- Now remove the rib cage.
- Cut the trachea and put it into a 50 ml conical tube containing 30 ml Ham's F12 medium supplemented with antibiotics, on ice. The medium will wash the trachea.
- In the next step, transfer the trachea into a 100 mm Petri plate containing 10 ml Ham's F12 medium supplemented with antibiotics.
- With the help of sterile forceps and surgical scissors, gently dissect away the connective tissue.
- To expose the lumen, cut the trachea along the vertical axis.
- In the next digestion step, the trachea is treated with 0.75% protease in M199 medium at 4 °C for 14 h.
- Now is the step of harvesting the ciliated cells. The harvesting is done by shaking the mucosa in an M199 medium enriched with 10% FCS and 1% penicillin-streptomycin.
- To minimize fibroblast contamination, allow the cells to settle for 1 h in a plastic Petri dish.
- At this point, while the fibroblasts remain adhered to the bottom of the Petri dish the resulting ciliated cells may remain clustered and float in the media.
- Collect resulting clusters of ciliated cells and wash them after 5 min of centrifugation at 220 × *g*, in an M199 medium.
- Resuspend the cells in an M199 medium.
- From each of the trachea(s), (5–10) ciliated cell culture samples may be obtained.

6.18 Isolation and Culture of Mouse Tracheal Epithelial Cells

This method was established by Lam et al. 2011 ***with a minor modification.***

6.18.1 Reagent Preparation for Isolation and Culture of Mouse Tracheal Epithelial Cells

Before commencing the isolation, prepare the following solutions:

- **Ham's F12 Medium Preparation**

  In 250 ml of Ham's F12 basal medium, add penicillin (100 $U \cdot ml^{-1}$), streptomycin (100 $\mu g \cdot ml^{-1}$), and fungizone (3 $\mu g \cdot ml^{-1}$). Store at 4 °C for up to 4 weeks.
- **0.5% Pronase Solution Preparation**

  In 10 ml Ham's F12 medium, add 15 mg pronase, antibiotics, and antimycotics. Make the solution freshly and keep on ice until use.
- **Collagen 1 Solution Preparation**

  Prepare collagen 1 solution at a concentration of 50 $\mu g \cdot ml^{-1}$ in 0.02 N acetic acid.

  Add 1.0 ml of this collagen solution into each well of a 12-well transwell plate (Corning).

Now, cover the wells with paraffin and incubate overnight at room temperature.

- **DNase 1 Solution Preparation**
  To 18 ml antibiotic containing Ham's F12 medium, add 2 ml, 10 $mg \cdot ml^{-1}$ bovine serum albumin (BSA) stock solution, and 10 mg crude pancreatic DNase I. Make 1 ml aliquots and store them at -20 °C until use.
- **Preparation of Ham's F12 Medium Containing Antibiotics with 20% Fetal Bovine Serum**
  For a 200 ml Ham's F12 basal medium, add 40 ml FBS, 100 $U \cdot ml^{-1}$ penicillin, 100 $\mu g \cdot ml^{-1}$ streptomycins, and 3 $\mu g \cdot ml^{-1}$ fungizone.

6.18.2 Prepare Mouse Tracheobronchial Epithelial Cell Basic Medium Containing Antibiotics

To 475 ml DMEM/F12 basic medium, add 7.5 ml (1 M) HEPES solution, 10 ml (200 mM) glutamine solution, 2 ml (7.5%) $NaHCO_3$ solution, 100 $U \cdot ml^{-1}$ penicillin, 100 $\mu g \cdot ml^{-1}$ streptomycin, and 3 $\mu g \cdot ml^{-1}$ fungizone.

6.18.3 Prepare Mouse Tracheal Epithelial Cell Medium with 10% FBS

- Take 45 ml mouse tracheal epithelial cell basic medium containing antibiotics.
- In this medium, add 5 ml heat-inactivated FBS to make it a 10% serum-containing medium.
- Like all other cell isolation procedures, a clean work surface area, including a laminar flow hood, is necessary to isolate mouse tracheal epithelial cells.
- Standardized small instruments as required for other cell isolation instruments, such as laminar flow hood, humidified CO_2 incubators, inverted microscope, disposable plastic pipette, culture ware, table-top cell centrifuges, and cold room, are necessary.

NB: To add the exact concentration of antibiotics/other chemicals for cell culture, one may need to check the company booklet from where the chemicals are sourced.

6.18.4 Protocol for Isolation of Mouse Tracheal Epithelial Cells

As discussed above, this method was established by Lam et al. 2011 ***with a minor modification.***

- Commercially available mouse strains such as C57Bl/6, male 6–8 weeks old were used by the authors.
- For the culture of a 12-well transwell plate, around six mice are needed that will yield 1.5–2.0) $\times$ 10^5cells/mouse.
- The mice were euthanized using a standard procedure such as CO_2–-induced necrosis or by injecting pentobarbital.
- Remove the skin with the help of clean surgical scissors and scalpel around the tracheal area and expose the trachea.

- The upper abdomen needs to be opened before cutting the sternum and removing the rib cage.
- Continue removing the tissue until the end of the trachea is exposed.
- Now, place the trachea into a 50 ml conical tube containing 30 ml Ham's F12 medium with antibiotics on ice. This will cleanse the trachea and minimize the contamination risk.
- In the next step, transfer the tracheal tissue to a sterile 100 mm Petri dish containing 10 ml Ham's F12 medium supplemented with antibiotics.
- With the help of sterile forceps and surgical scissors, gently dissect the connective tissue.
- Expose the lumen-cut trachea along the vertical axis.
- Take a 50 ml tube, add 10 ml (0.15%) pronase solution and now transfer the trachea into it and incubate overnight at 4 °C.
- On the next day (after ~24 h), gently rock the tube 10–12 times and then let it stand for (30–60) minutes at 4 °C.
- Add 10 ml Ham's F12 medium containing 20% FBS and antibiotics to the tube and rock 12 times.
- Get the 3, 15 ml conical tubes prepared to contain 10 ml Ham's F12 medium having antibiotics and 20% FBS.
- The trachea is removed from the pronase solution before being placed in an ice solution.
- In the next step, the trachea is transferred to the first conical tube containing Ham's F12 and then inverted 12 times. Repeat this process twice.
- Henceforth, take one 50 ml tube and merge the pronase solution with three supernatants.
- Discard the remaining tissue.
- Centrifuge the collected supernatant at 1400 rpm (390 × *g*) for 10 min.
- Discard the supernatant.
- Resuspend the pellet in a 1 ml DNase solution ((100–200) µl/trachea) and incubate for 5 min on ice.
- Again centrifuge at 1400 rpm (390 × *g*) for 5 min at 4 °C.
- Discard the supernatant.
- Finally, resuspend the cell pellet in an 8 ml MTEC medium containing 10% FBS.
- Transfer the cell suspension on Primaria plates (Falcon).
- Incubate the Primaria plates at 37 °C in an atmosphere of 95% air and 5% CO_2 for 5 h.

NB: The following steps are used for the negative selection of fibroblasts.

- Take the plates to retrieve the cell suspension.
- Rinse the plates with 4 ml MTEC containing 10% FBS.
- Take a 50 ml conical tube and pool the cell suspension into it.
- **NB:** Put 1 ml separately for cytospin and cell counting.
- In a table-top centrifuge, spin at 5000 rpm for 5 min.
- Remove 500 µl and resuspend the pellet in the remaining supernatant.

- For cell counting by Trypan Blue vital staining method, use 100 μl cell suspension.
- Conserve four aliquots of 100 μl each for cytospin analysis.
- Spin the remaining 15 ml cell suspension at 1400 rpm (390 × *g*) at 4 °C for 10 min.

6.18.5 Mouse Tracheal Epithelial Cells at Air–Liquid Interface: Propagation and Differentiation

Retinoic Acid Stock Solution Preparation

- Make a 5 mM stock solution of retinoic acid (mol. wt. 300.44 g μg/mol) in 95% ethanol.
- Store in a foil-wrapped tube at −80 °C.

NB: It is a light-sensitive molecule. So, preparation must be done in dark. It is herewith referred to as stock solution A.

Stock Solution B Preparation

- For the preparation of 50 μM stock solution B, add 50 μl stock A, 500 μl BSA solution (100 mg/ml), and 49.5 ml **HBSS**.
- Store in a foil-wrapped tube at −80 °C for up to 4 weeks.

6.18.6 Mouse Tracheal Epithelial Cells Proliferation Medium with Retinoic Acid Preparation

To prepare mouse tracheal epithelial cells basal medium (**MTEC**), add the following:

47.5 ml MTEC with antibiotics
2.5 ml FBS (heat inactivated)
1 ml retinoic acid stock B.
250 μl insulin solution (2 mg/ml insulin in 4 mM HCl).
250 μl epidermal growth factor solution (5 μg/ml EGF in HBS containing 1 mg/ml BSA) 200 μl bovine pituitary extract (15 mg/ml in HBS containing 1 mg/ml BSA).
50 μl transferrin solution (5 mg/ml transferrin in HBS containing 1 mg/ml BSA.
50 μl cholera toxin solution (100 mg/ml in HBS containing 1 mg/ml BSA).
Filter-sterilize the medium and use it within 2 days.
Remove the collagen solution from the transwell plates.
Wash with sterile PBS twice.
Centrifuge the content.
Resuspend the cell pellet in ~500 μl.
Plate 7.5×10^4 -1.0×10^5 cells per well.
In the basal compartment of the transwell plate, add a 1.5 ml proliferation medium.
The submerged MTEC cultures should be incubated at 37 °C in a humidified incubator containing 95% air and 5% CO_2 for (7–10) days.

Change the medium after 3 days.
Thereafter, change the medium every 24 h.
Monitor cultures by visual inspection and measurement of transepithelial cell resistance (EVOM Ohm voltmeter, World Precision Instruments, Sarasota, FL).
When cells appear confluent and epithelial resistance reaches 1000 Ω/cm^2, they are ready to differentiate.

6.18.7 Mouse Tracheal Epithelial Cells Basal Medium Preparation

Take a sufficient quantity of MTEC and add 2% NuSerum.

- Add retinoic acid stock B to a final concentration of $\mathbf{1 \times 10^{-7}\ M}$ before using.
- Prepare fresh and use within 2 days.
- Allow cells to differentiate for (10–14) days by removing the apical medium and replacing the basal medium with a 750 μl MTEC basal medium containing 2% NuSerum and retinoic acid.
- Change the basal medium and wash the apical side with MTEC containing 2% NuSerum every 24 h.

6.19 Isolation and Culture of Pulmonary Alveolar Epithelial Cells

- The human pulmonary alveolus contains two kinds of epithelial cells. They are alveolar type 1 epithelial **(ATI)** cells that cover around (90–97)% alveolar surface area and alveolar type II epithelial **(ATII)** cells that cover around (3–10)% alveolar surface area.
- AT-II cells become AT I cells when AT I cells are injured or die.
- Therefore, **AT-II cells act as progenitors for AT I cells**.

6.19.1 Identification and Characterization of Alveolar Type 1 Epithelial Cells

AT I cells cover (90–97)% alveolar wall.

6.19.2 Morphology and Structure of Pulmonary Alveolar Type I Cells

AT I cells can be identified based on their morphology, while being observed using an electron microscope with the following characteristics:

Large squamous epithelial cells
Flattened nuclei
Few mitochondrion
Few cellular inclusions
No lamellar bodies

6.19.3 Metabolic Activity and Cell Division of Pulmonary Alveolar Type I Cells

Metabolically inactive, rarely divide or may not divide at all.

6.19.4 Functions of Alveolar Type 1 Epithelial Cells

AT1 cells provide a barrier function to alveoli and help in gas exchange.

NB: Isolation and culture of ATI cells are difficult for the following reasons:

- They have very thin cytoplasmic extensions.
- They form very tight intercellular junctions.
- They are metabolically inactive.
- They rarely divide or do not divide at all.

6.19.5 Identification and Characterization of Alveolar Type 1I Epithelial Cells

AT-II cells cover (3–10)% of the alveolar wall.

6.19.6 Morphology and Structure of Alveolar Type 1I Epithelial Cells

Small cuboidal epithelial cells.

A large number of lamellar bodies with a histology can be confirmed using electron microscopy [via modified papanicolaou or nile red (a fluorescent dye) staining].

Abundant mitochondrial presence.

6.19.7 Metabolic Activity and Cell Division

- Highly metabolically active and actively dividing cells.
- AT-II cells are readily activated by TNFα and produce various cytokines, SP-C, and intercellular adhesion molecule 1 (ICAM-1).

6.19.8 Functions of Alveolar Type II Epithelial Cells

Produces surfactants and pulmonary host defense proteins.

6.19.9 Culture of Alveolar Type II Epithelial Cells

- The isolation of rat alveolar type II (AT-II) epithelial cells was first published in 1974.
- Since then, a large number of studies isolated and cultured AT-II epithelial cells from various mammalian species including humans.
- Some important methods in this field were established by Dobbs 1990; Corti et al. 1996; Wang et al. 2007; and Ballard et al. 2010.
- In 1996, *Rannels and colleagues* described the culturing of primary rat AT-II epithelial cells at various stages of differentiation.

The following is a protocol for isolation and culture of human primary alveolar type II epithelial cells:

6.19.10 Specimens Collection and Isolation of Alveolar Type II Epithelial Cells

The protocol presented here for isolation and culture of human primary AT-II epithelial cells was published by Mao* et al.** 2015. ***This method of isolation was

established by the adaption and modification of several previous protocols. Here is the step-by-step protocol:

- Obtain the specimen of distal normal lung tissue (6–10) g from patients undergoing lung resection under sterile conditions.
- The lung specimen should be cut into a 1 cm^3 cube.
- Extensively wash the pieces with Hanks' balanced salt solution (**HBSS**).
- With the help of scissors, mince the lung pieces (0.5 mm^3) and transfer them to a sterilized beaker containing 50 ml BSS.
- Mix gently.
- Filter it by passing through a mesh (150 μm) cell strainer (BD Falcon, San Jose, CA).
- Incubate the lung tissue for 45 min in a mixture of proteolytic enzymes containing 3 ml trypsin (10,000 U/ml) and 300 µl elastase (5.1 U/ml) in a shaking water bath at 37 °C. Add DNase I at 2 ml (10,000 U/ml) for 15 min.
- Stop the proteolytic digestion using 40 ml inhibition solution (30 ml DMEM: F-12, 1:1, 10 ml FBS and 1 ml DNase I 10,000 U/ml).
- Further, dilute the digested suspension by adding 400 ml HBSS and thorough whiffling for 10 min using a Pasteur pipette.
- Now, filter the suspension using the cell strainers at the size of (150, 75, and 40) μM in tandem to collect the crude cell extract.
- In the next step, centrifuge the suspension at 400 × g for 10 min at room temperature.
- Resuspend the cell pellet in 100 mm Petri dishes containing 10 ml of each adhesion medium for macrophages and fibroblasts.
- The medium constitutes the following: 1:1 mixture (22.5 ml each) of DMEM-F-12 and small airway epithelial cell growth medium, SAGM; 5 ml FBS and 1 ml DNase I 10,000 U/ml.
- Incubate the cells at 37 °C for 150 min.
- Early attachment of macrophages and fibroblasts with the cell culture containers was noticed.
- Epithelial cells attach late. This characteristic distinction helps in separating the epithelial cells from macrophages and fibroblasts.
- Centrifuge and resuspend the cell pellets in 3 ml DMEM-F12 and layer the crude cell suspension onto a (1.040–1.089) g/ml discontinuous Percoll gradient.
- A solution of 40 ml light gradient (1.040 g/ml) containing 4 ml PBS, (10 × 12.55) ml Percoll solution (MP Biomedical, Solon, OH), and 23.45 ml distilled water, is preserved.
- A solution of 40 ml heavy gradient (1.089 g/ml) containing 4 ml PBS (10 × 25.96) ml Percoll solution (MP Biomedical), 10.04 ml distilled water, and one drop of phenol red is obtained.
- Take 15 ml centrifuge tubes in which 3 ml from each solution is gradually added to 5 ml FBS. Subsequently, conduct the centrifugation at 300 × g for 20 min at 4 °C, with the aid of a swing-out rotor. This results in the formation of a dense

layer of AT-II cells along with the interface between the two Percoll gradient layers.
- Conduct deceleration via turning off the power as the designated centrifugation duration is neared.
- Relocate the intensified cell pellets (of AT-II cells) in a 15 ml centrifuge tube already having 13 ml BSS. Perform centrifugation twice at 300 × *g* till 10 min at room temperature.
- Resuspend the pellets in a 2 ml HBSS and attempt a magnetic bead-assisted separation to get rid of any kind of macrophage contamination (Anti-CD^{14} MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany).
- Subject the isolated AT-II cells at 300 × *g* centrifugation for 10 min at room temperature. Thereafter, resuspend the cells in a 2 ml SAGM, carrying 1% FBS. Thereafter, incubate the cell mixture in 60 mm culture dishes at 37 °C for 60 h, changing the medium every 24 h.
- Subject the cells to subculturing, either in SAGM or DMEM having either 1% or 10% FBS till 21 days. The 10% FBS, in general, exhibits a greater growth.

6.19.11 Limitations of the Culture of Pulmonary Alveolar Type II Epithelial Cells

- Several investigators, including Campbell et al. 1999 ***and*** Meleady et al. 1998**,** have suggested an important feature of isolated AT-II epithelial cells is their loss of specific features within days of in vitro culture and the acquisition of AT I epithelial cells characteristics.
- This process is highly dependent on cultural conditions and is termed **trans-differentiation**.
- Of note, in vitro trans-differentiation is at least partially reversible, but it is not known whether the reversibility of AT-II epithelial cell differentiation into AT I epithelial cells is a potential regulatory mechanism in vivo.

6.20 Isolation and Culture of Mammalian Mammary Epithelial Cells.

6.20.1 Basic Structure of Mammary Gland

Some basic aspects of the mammary glands are as follows:

In mammals, mammary glands (breast) produce milk and also act as secondary sexual organs.

- The human breast comprises a specialized branching duct network that culminates in clusters of smaller ductules. Together, these comprise the terminal ductal lobular units (TDLUs).
- Of note, TDLU may comprise (30–50) acinar cells grouped as a lobule along with their associated ducts.
- Interestingly though, acinar cells are the smallest functional structures in a breast.

- A terminal duct, in general, arises from each acinar cell. The branching ducts besides acini prevail at the terminus of each ductal system.
- The primary function of the acini is to secrete milk.
- Milk secreted by the acinar cells passes through the lobule and duct to reach the nipple.
- The cellular structures predominating the breast are epithelial cells. Significantly, the mammary epithelium is quite distinct contrary to several other human body tissues as it persists to develop right after birth. Major events in course of this development comprise vigorous remodeling with branching cycles, acini generation, and dissolution of epithelial structures amidst puberty, pregnancy, lactation, and involution.

The branching ducts and acini are composed of three types of cells, which are as follows:

Mammary stem cells
Myoepithelial cells
Luminal epithelial cells

The followings are a brief discussion of the three types of human mammary epithelial cells.

6.20.2 Mammary Stem Cells

- Mammary stem cells (**MaSCs**) are small progenitor cells located at the basal or outer layer of the duct/ductules/acini.
- MaSCs are located at the top of the epithelial hierarchy and have unique traits, including self-renewal and multidirectional differentiation.
- These cells proliferate and differentiate into myoepithelial and luminal progenitor cells that further differentiate into myoepithelial cells or luminal epithelial cells.

6.20.3 Myoepithelial Cells

- The outer layer of the ductal/lobular/acini structures of the mammalian breast is located at the surface of the basement membrane and is composed of myoepithelial cells.
- These cells are flattened and elongated in their morphology.
- These cells contain numerous myofibrils (the contractile units), the absence of rough-surfaced endoplasmic reticulum (rough ER), and lipid droplets.
- Milk flow into the ducts is driven by the contractions of the myoepithelial cells (Fig.11).

6.20.4 Luminal Epithelial Cells

- The inner layer of the ductal/lobular/acini structure of the mammalian breast is composed of luminal epithelial cells, with apical microvilli.

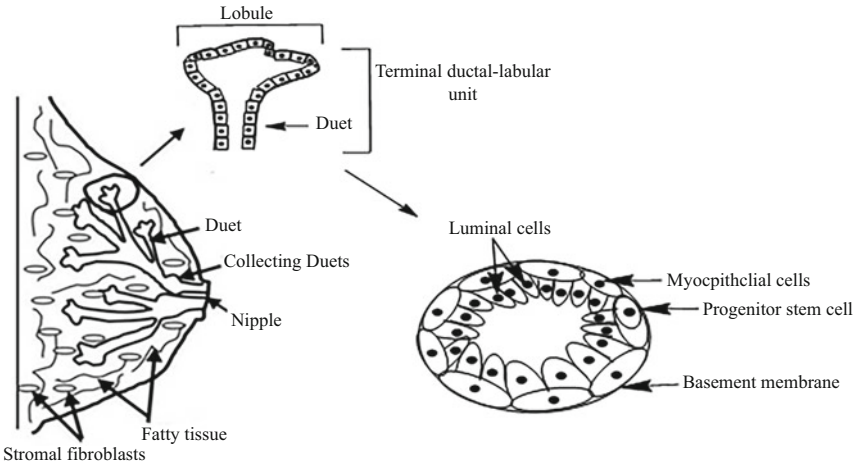

Fig. 11 Schematic depiction of the location of various types of epithelial cells in the human breast. (Dimri et al. 2005; Breast Cancer Research] with modifications)

- The luminal lineage can be further subdivided into ductal and alveolar luminal cells that line the ducts and constitute the alveolar units arising during pregnancy (Fig.11).
- These cells are arranged radially with tight junctions and narrow widths. These cells secrete milk.

6.20.5 Isolation, Purification, and Characterization of Mammary Epithelial Cells

- A large number of studies have established the isolation and culture methods of epithelial cells from mammalian breast tissues.
- Notable examples in this field include publications by Shipitsin et al. 2007; Labarge et al. 2013; and Raouf and Sun 2013.
- In 2015, *ZubeldiaPlazaola* et al compared the different isolation and culture methods of mammalian breast luminal and myoepithelial cells.
- The cell yield and viability of the mammary epithelial cells isolated by various methods depend on the following factors:

  Mechanical Handling

  Whether or not to discard adipose tissue and the specific size of a piece of tissue.

  Proteolytic Absorption

  This method critically depends on digestion time. Type and concentration of digestive enzymes, viz., collagenase/hyaluronidase or a stoichiometric blend of the two.

  Separation of Cell Portion

  This is accomplished using sequential filtering or differential centrifugation.

  Final Cell Isolation

  This is done either using immunogenetic beads or via sorting.

- Primary cultures suffer from certain restrictions such as the episodes of senescence, once (10–40) population doublings have happened. Thereby, these cultures are often not useful for long-term requirements.
- Studies by Garbe et al. (2009) established that the medium configuration and the inclusion of Rho-associated protein kinase (ROCK) hinder or prolong the senescence happening after digestion. Such changes or hallmarks result in improved cell proliferation by preventing anoikis (perishing of cells due to loss of attachment).
- Technical snags demonstrated to date have a significant impact on cell viability and yield, and impede the primary cells culture, suggesting a requirement to streamline the technology for apt segregation of epithelial and myoepithelial cells.

6.20.6 Materials for Isolation and Culture of Mammalian Mammary Epithelial Cells

Materials needed for isolation and culture of mammary (breast) epithelial cells are the same as for isolation and culture of other mammalian cells. Materials specifically needed are as follows:

- DMEM: F12 in 1:1 ratio with antibiotics and antimycotics
- Bovine serum albumin (BSA)
- Collagenase type IV
- Hyaluronidase
- ROCK inhibitor or Y27632
- Epithelial and organoid culture medium: M87A formula previously described (Garbe et al. 2009)

6.21 Isolation and Culture of Human Mammary Epithelial Cells

This protocol is developed from the publication by Jin et al. 2018 ***with minor modifications. The following steps describe the protocol:***

- All the tasks must be completed aseptically in a laminar flow hood.
- Collect normal human breast tissues from surgical specimens with written informed consent from the patient and store them at 4 °C before processing. The breast tissue must be processed within 3 h of collection and discarded if it is not processed more than 3 h after collection.
- The tissue is minced or cuts into 1 mm^2 pieces.
- Now the breast tissue is treated with collagenase and hyaluronidase mixture overnight at 37 °C.
- Following overnight treatment with enzymes, the mixture is filtered using a nylon strainer.
- Collect the flow-through fractions and centrifuge the fractions at 1200 rpm for 10 min to obtain cell pellets.

- Use Accumax to further dissociate the cells and obtain single cells.
- Resuspend the cells in F-medium. F-medium contains Dulbecco's Modification of Eagle's Medium: Ham's F-12 (1:1) with 2 mM L-glutamine, 5% FBS, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 8.4 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, and 24 μg/ml adenine with 10 μM Y-27632.
- Now, seed the cells on a layer of 50% confluent irradiated or mitomycin-treated 3 T3-J2 feeder cells for expansion. Maintain all cultures at 37 °C and 5% CO_2.
- To reach 80–90% confluence in 6-well plates, the initial colony expansion may take 1–2 weeks.
- Passage cells with 1:10 splitting.
- Now the cells take lesser time (within 4–6 days) for (80–90)% confluence.
- These cells can be passaged with accutase (Innovative Cell Technologies) to avoid the negative impact of enzymatic conditions.

6.22 Isolation and Primary Culture of Human Mammary Epithelial Cells

The presented protocol is the one proposed by Kothari et al. 2003 ***with minor modifications and consists of the following steps:***

- Collect the breast tissue following surgery.
- Mince or cut it into small pieces.
- Mince the breast tissue and digest overnight at 37 °C using type 1A collagenase (1 mg/ml) in RPMI-1640 plus 5% FCS and 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml polymixin B, and 2.5 mg/ml amphotericin B.
- Now decant off the fat tissue.
- Use the medium to wash the remaining organoids and cells three times.
- Wait for 20 min so that the organoid settles down.
- Remove the supernatants and resuspend in RPMI-1640 containing 1% FCS.
- Complete another two rounds of sedimentation.
- Now, digest the pellet with trypsin/EDTA (0.05/0.02% in PBS) plus 0.4 mg/ml of DNase I for (15–30) minutes at 37 °C.
- Terminate the reaction by adding cold RPMI plus 10% FCS.

6.22.1 Purification of Epithelial Cells

- The isolated epithelial cells are purified via immunoaffinity using super-paramagnetic, polystyrene beads (Dynal Ltd., New Ferry, Wirral, UK) coated with a mouse IgG1 monoclonal antibody (MAbBer-EP4) specific for two (34 kDa and 39 kDa) glycopolypeptide membrane antigens as described by Latza et al. 1990.
- Culture the purified cells in BCM (DMEM: F-12 (1:1), supplemented with 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml polymixin B, 2.5 mg/ml amphotericin B, 5 mg/ml insulin, 10 mg/ml

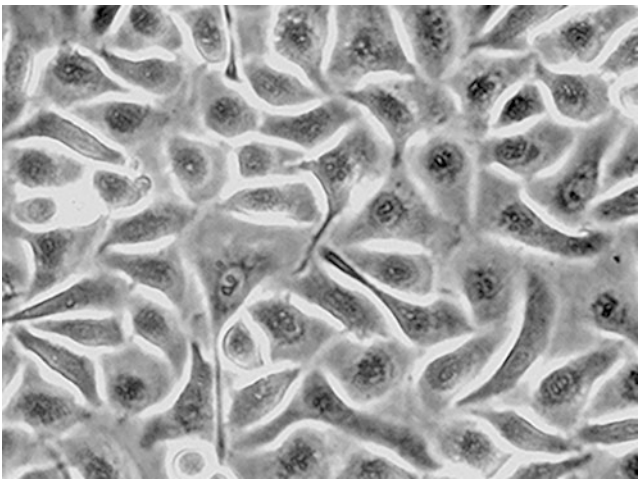

Fig. 12 Phenotypic growth pattern of cultured mammary epithelial cells (100% confluent) as observed through an inverted microscope

apotransferrin, 100 mM ethanolamine, 1 mg/ml hydrocortisone, 10 ng/ml EGF, and 10% FCS as described by Gomm et al. 1995.

- Figure 12 depicts the phenotype morphology of cultured mammary epithelial cells in a 100% confluent state.

6.23 Isolation and Culture of Human Mammary Epithelial Cells

The presented protocol follows the method of Stampfer and Yaswen 1993 ***with minor modifications. A brief description of the protocol is as follows:***

6.23.1 Dissociation Buffer

Tissue mix medium: Mammary epithelial cell growth medium (MEGM) without additives from Cambrex or Ham's F12 + insulin (10 μg/ml**),** penicillin (100 U/ml), streptomycin (100 μg/ml), polymixin B (50 U/ml), and fungizone (3 μg/ml). Store at 4 °C.

6.23.2 Preparation of Enzyme Solution

- For preparing collagenase solution, dissolve 1500 U/ml collagenase in the appropriate amount of tissue mix medium at 37 °C.
- Filter the collagenase solution through a 500 ml bottle top filter.
- For preparing hyaluronidase solution, dissolve 1000 U/ml hyaluronidase in the appropriate amount of tissue mix medium at 37 °C.
- Filter-sterilize just like in the case of collagenase solution preparation.
- Aliquot 30 ml into 50 ml conical tubes, and store at -70 °C for up to 1 year. (This is a 5X solution).

6.23.3 Digestion and Isolation of Mammary Epithelial Cells

As discussed in the above section, this method was established by Stampfer and Yaswen 1993***, with minor modifications.***

- Obtain human mammary tissue as discarded material from surgical procedures.
- Place material in sterile containers containing sterile buffer or tissue mix medium with 10% FBS for up to 72 h.
- Separate the epithelium from the stromal matrix in sterile 150 mm Petri dishes using a combination of a sterile scalpel, forceps, and scissors.
- The epithelium appears as white strands embedded in the stromal matrix. Dissect these areas, scraping away the grossly fatty material.
- Prepare a 50 ml conical tube for the tissue by adding 5 ml FBS and an 18.3 ml tissue mix medium.
- Transfer the minced epithelium-containing tissue into a conical centrifuge tube.
- Fill the tube to full volume with enzyme solution (10 ml), leaving only a small air space to allow gentle mixing during rotation.
- Place tubes on a tube rotator and rotate overnight at 37 °C.
- Centrifuge tubes at 600 × g for 5 min.
- Discard the supernatant fat and medium.
- Dilute a small aliquot of the pellet in a medium to microscopically examine the degree of digestion.
- Digestion is complete when microscopic examination shows clumps of cells (organoids) with ductal, alveolar, or ductal–alveolar structures free from the attached stroma.
- If the tissue is not fully digested, resuspend the pellet in fresh tissue digestion medium at approximately the same pellet to medium ratio.
- Reincubate with rotation at 37 °C for additional (4–12) hours.
- Recentrifuge the tubes and recheck the pellet.
- If digestion is still not complete, add fresh digestion medium and incubate again till overnight.
- The concentration of enzymes in the digestion medium can vary according to the needed extent of digestion.
- Washing and freezing buffers: CPMI: Add 15 ml FBS and 10 ml DMSO to 75 ml, 1:1 DMEM and F12 mixture; shake gently and store indefinitely at −20 °C.
- Wash the remaining organoids and single cells with medium at least thrice.
- To enrich the preparation for ductal and lobular elements and to eliminate free blood cells, fibroblasts, and endothelial cells, complete three (30–60) minutes of sedimentation at 1000 × *g* (on the bench-top centrifuge).
- Remove the supernatant containing stromal cells.
- A pellet of (5–10) ml organoids should be obtained from each preparation.
- Pellet the organoids by centrifugation at 600 × *g* for 5 min and remove the supernatant.
- Add 1 ml CPMI for every 0.1 ml pellet.
- Seed a Petri dish for each tube by placing 0.1 ml resuspended material into 35 mm dishes drop by drop to fill and cover the dish surface.

- Disperse the organoids in the dish by gently rocking the dish to spread out the medium.
- Let it sit for 1 min and then add 1 ml growth medium to the dish.
- Incubate at 37 °C and check for attachment and sterility the following day.
- Store fractions in liquid nitrogen until use.

6.23.4 Identification of Epithelial Cells Based on Phenotypes

Shape of the Epithelial Cells

The epithelium is classified into various types based on the shape of the superficial cells and the number of cell layers.

Squamous Epithelium

Flattened plate-like cells**.**

Cuboidal Epithelium

Approximately same height and width.

Columnar Epithelium

Cells have a greater height than width.

Diameter of the Epithelial Cells

Epithelial cells show a range of diameters, from (8 to 21) microns with 97% of measurements lying in the (9–17) micron range. The mean cell size is 12.7 microns.

6.23.5 Identification of Various Epithelial Cells Based on Marker Proteins Expression

Although epithelial cells are located throughout the body, in this section we describe the isolation and primary culture of epithelial cells only from the trachea, alveoli, and mammary glands.

Epithelial marker proteins of these organs are described as follows.

Tracheal Epithelial Cells Marker Proteins

Both human and mouse tracheal epithelial cells are characterized by the expression of the following molecules:

- Cytokeratins include cytokeratin 5 (Krt 5) and cytokeratin 14 (Krt 14).
- Mucin proteins include MUC16 and MUC5AC.
- Transcription factors include Trp63.

Alveolar Type I Epithelial Cells Marker Proteins

AT1 cell markers include T1α protein, aquaporin 5 (AQP-5), and caveolin-1.

Alveolar Type II Epithelial Cells Marker Proteins

Aquaporin 3, surfactant proteins (SP-A, SP-B, SP-C, and SP-D), CK-8, KL-6, α ENaCRTII 70, and LB180.

Mammary Stem Cells Marker Proteins

- In 2007, ***Shipitsin*** **et al.** identified CD44, CD29, CD49F or integrinα-6, EpCAM, and CD24 as mammary stem cell markers in the epithelium.
- In 2007, ***Ginestier*** **et al** reported that normal and malignant human mammary stem cells express aldehyde dehydrogenase (ALDH).
- In 2007, ***Chen*** **et al** identified Lin^{-} $Procr^{+}$ $CD24^{+/med}$ $CD29^{hi}$ $CD49f^{hi}$ $Sca1^{low/-}$ and $Lin^{-}CD49F^{+}EpCAM^{neg-low}$ or $CD10^{+}$ as markers of mouse and human breast stem cells, respectively.

Mammary Myoepithelial Cells Marker Proteins

- Myoepithelial cells are characterized by the expression of cytokeratin 14 (CK 14), alpha-smooth muscle actin (α-SMA), and vimentin.
- Another important marker protein present in myoepithelial cells is CD10 (Fu et al. 2014; Batistatou et al. 2003).

Mammary Luminal Epithelial Cells Marker Proteins

- Several proteins are recognized and well-characterized as markers of luminal epithelial cells.
- The general luminal epithelial cell markers are cytokeratin 8 (CK8), cytokeratin 18 (CK-18), cytokeratin 19 (CK-19), Mucin 1 (Muc 1), and the epithelial cell adhesion molecule (EpCAM).
- The K8 and K18 are reported as a pair of keratin filaments, restricted to luminal cells in mouse and human mammary glands.
- In 2014, *Fu* et al. identified CD227, EpCAM, CD44, and CD24 in luminal epithelial cells.

Differences in Keratin Expression Between Luminal Epithelial Cells and Myoepithelial Cells

- As discussed in the previous section, a protective structural protein is expressed by various types of epithelial cells. Several keratin proteins are expressed by epithelial cells and hair. While in luminal epithelial cells, keratin 8 and keratin 18 are expressed, in the mammary epithelial cells, keratin 19 appears to be associated with the most mature, well-differentiated cells.
- In addition, luminal epithelial cells also express **mucin** and **PEM,** which can be detected by their respective specific antibodies.
- For basal epithelial cells, the marker proteins include **vimentin, keratins, smooth muscle actin,** and acute lymphoblastic leukemia antigen (**CALLA**).
- Myoepithelial cell identification in a basal cell population requires evidence for the presence of contractile myofibrils or oxytocin receptors.

- p16 expression is one of the important post-selection characteristics. The cells maintain certain expressions of keratins 5, 7, and 14 while acquiring increasing keratins 8, 18, and PEM expressions.

6.23.6 Utility of Epithelial Cells

- Present at the boundary and lining of all cavities, organs, and exterior–interior surfaces, epithelial cells protect organs and help in maintaining their structural and functional integrity.
- Epithelial cells are involved in the transportation, absorption, secretion, lubrication, and movement of organs.
- To understand the structure–function relationships of epithelial cells in specific organs, it is necessary to culture them.
- While epithelial cells can be isolated and cultured from any of the abovementioned organs or tissues, the source of the two most widely studied epithelial cells is the respiratory tract and the female breast.
- Approximately 80% of mammalian cancers are of epithelial origin.

7 Conclusions

This chapter describes the isolation and culture of endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. Depending upon the type, these cells are present in both the vascular and nonvascular regions of a mammalian body. While endothelial cells are present only in the vascular bed (micro- and macrovascular) of the body, smooth muscle and cells and fibroblasts are present both in the vascular and nonvascular regions of the body and epithelial cells are present in both outer and inner body surfaces without any blood supply (vascular bed). Each of these cells has a specific distinct morphology and phenotype through which these can be identified and confirmed. For example, endothelial cells have **cobblestone** morphology, smooth muscle cells are **spindle-shaped,** fibroblasts are **large, flat, elongated (spindle-shaped) or round cells with extensions radiating out from the ends of the cell body,** and finally, epithelial cells have three distinct morphologies such as **flattened plate-like** (squamous epithelium), **approximately same height and width as cubes** (cuboidal epithelium) and the cells having **greater height than width as column** (columnar epithelium**)**. These cells can also be separated and distinguished from each other based on their specific size, marker protein expression, required culture medium, and doubling time. The isolation and culture procedure of these four cell types described in this chapter is based on the personal research experience of the authors as well as modification of the various well-established techniques by other eminent researchers. Based on the numerous physiological and pathophysiological roles played by these four types of cells, learning about the isolation and culture of endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells is undoubtedly essential.

8 Cross-References

- Culture of Continuous Cell Lines
- Culture of Neuron and Glia Cells
- Primary Culture of Immunological Cells
- Stem Cell Culture and Its Applications

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Primary Culture of Immunological Cells

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https://doi.org/10.1007/978-981-19-1731-8_9-1

Abstract

This chapter begins with a brief description of the mechanisms associated with maintaining immunity (protection against disease-causing pathogens) in the mammalian body. All the immunological cells whether involved in innate (inherited from parents to offspring) or acquired immunity (newly developed in response to pathogen) originated from hematopoietic stem cells (**CD**34$^+$) within the red bone marrow. The immunological cells are not only present in the body fluids (e.g., blood, lymph, etc.), but also in the **primary lymphoid organs** such as

red bone marrow and **thymus** as well as **secondary lymphoid organs** such as the **spleen, lymph nodes**, mucosa-, and gut-associated lymphoid organs (**MALT and GALT**), respectively. This chapter describes the isolation of various primary and secondary lymphoid organs such as the red bone marrow, thymus, spleen, and lymph nodes as well as different immunological cells present in these organs and body fluids such as blood. Isolation, purification, enrichment, and culture of the innate immunological cells including **monocytes and tissue macrophages** from various regions of a mammalian body and other immunological cells, viz., **dendritic cells** and **natural killer (NK) cells,** are discussed. In addition, isolation, purification, enrichment, and culture of acquired immunological cells, i.e., both **B cells** and various **T cells (T helper, T cytotoxic, T regulatory),** and **γδ T cells,** as well as isolation and culture of a **natural killer like T cells** (**NKT), an invariant natural killer like T (iNKT) cells,** and **co-culture of various cells,** are described. The chapter ends with a very brief note on the significance of isolation and culture of these different mammalian immunological cells.

Keywords

Euthanasia of Animals · Isolation of Primary and 2ndary Lymphoid Organs · Isolation and Characterization of Eosinophil and Neutrophils · Isolation and Culture of Monocytes and Macrophages · Isolation and Culture of Dendritic Cells · Isolation and Culture of gamma/delta T Cells · T helper (Th) and T cytotoxic (Tc) CellsIsolation and Culture · T regulatory (Treg) Cells isolation and Culture · NK and iNKT Cells Isolation and Culture · Co-culture of Immunological Cells · Splenic B Cells Culture · Isolation of Mast Cells

1 Introduction

This chapter describes the isolation and primary culture of cells from hemato-immunologic organs. Live mammalian cells are commonly harvested directly from the live tissue or organs upon the sacrifice of an anesthetized animal, followed by subsequent in vitro culturing in a synthetic, sterile medium. This type of primary cell culture from normal tissues is mainly intended to study the cellular function in terms of genes and proteins, normally present in the cells. Cells from genetically altered **knockout** or **transgenic animals** display either the **absence** or **overexpression** of specific proteins in a relatively normal cellular background.

Primary culture of cells obtained from blood or organ(s) of human patients provides a unique opportunity to study the structural-functional relationship of various genes and proteins present in these cells so that the underlying cause and recovery of the disease can be understood. In particular, primary cell cultures from either animals or patients are of prime importance for studying the immune system compared to any secondary culture or culture of immortalized cell lines due to a poor resemblance with the natural normal functioning cells present in the in vivo system.

The chapter commences with the basic concept of innate and acquired immunity, succeeded by a brief discussion of immunological organs. While the immunological cells originate and mature in the primary lymphoid organs, i.e., red bone marrow and thymus, the secondary lymphoid organs, i.e., spleen, lymph nodes (LNs), mucosa, and gut-associated lymphoid tissue (MALT and GALT) are the locations where immunological cells encounter antigens. Thereafter, these cells are further proliferated, differentiated, and finally stocked up to protect against the possible future attacks by exactly similar antigens.

Following the discussion of lymphoid organs, we introduce the innate and acquired immunological cells involved in mammalian immunity manifestation. All the immunological cells originate from hematopoietic stem cells ($CD34^{+}$) present in the red bone marrow, subsequently proliferating and differentiating into myeloid and lymphoid progenitor cells. The myeloid progenitor cells further proliferate and differentiate into non-immunological cells such as RBC and platelets and immunological cells such as macrophages, eosinophils, neutrophils, basophils, mast cells, and dendritic cells. The lymphoid progenitor cells further proliferate and differentiate into lymphocytes (B/T cell), natural killer (NK) cells, dendritic cells, and NKT cells. The structure and function of all these cells are discussed.

Henceforth, the isolation of various immunological organs such as the spleen, LNs, thymus, and various immunological cells from the blood and different (primary and secondary) lymphoid organs is discussed. At the end of this chapter, the culture of various immunological cells is described comprising macrophages from various sources, such as dendritic cells, NK cells, NKT cells, neutrophils, eosinophils, mast cells, B cells, Th1 cells, Th2 cells, and the cytotoxic T cells. Co-culture of some cells is also mentioned here. Additionally, some assays such as ^{51}Cr release assay and infection of splenic macrophage culture by leishmanial parasites are discussed. To summarize, this chapter helps the readers acquaint themselves with the basic knowledge of immunity, immunological organs, innate and acquired immunological cells, their isolation, and culture (Steinhauser et al. 2014; Strober 2001).

2 Immunity: Innate and Acquired

- **Immunity** is the protection of a body against disease-causing pathogens/microorganisms (e.g., mycoplasma, bacteria, protozoa, fungus, etc.) and foreign elements (e.g., pollens, viruses, etc.). To be effective, the immune system should be able to identify the **foreign or non-self and disease-causing** particles, either as a **part of our body or entirely foreign**.
- The word **"Self"** here, implies **the** molecules including various proteins that are produced by somebody's own body (**endogenously generated**) and therefore the immunological cells in this particular individual do not react with the self molecules.
- On the other hand, "**Non-self"** refers to proteins or molecules of disease-causing pathogens such as viruses, mycoplasmas, bacteria, fungi, protozoans, and others. Upon exposure to the human body, these externally sourced molecules may cause

multiple diseased conditions. Thus, our immunological cells recognize these moieties as **foreign or non-self** molecules, interact with them and destroy them entirely.

- Circulating immunological cells (B and T cells) enter the thymus, the primary lymphoid organ of our body, and interact with self-major histocompatibility complex (**MHC I** and **MHC II**), expressed on the surface of thymus cortex epithelial cells. Thereafter, these facilitate the lesion between self and non-self, tolerate self-cells containing self-MHC (called self-tolerance), and interact with non-self or foreign molecules expressed on non-self-cells to destroy them.

So, the basics of immunity lies are about the ability of immunological cells lesion to distinguish between self and non-self and tolerate self-cells and molecules besides destroying the disease-causing non-self-cells and molecules.

3 Types of Immunity

Immunity can be divided into two types, viz., innate and acquired. Table 1 comprises the basic differences between innate and acquired immunity.

The following paragraphs briefly describe the innate and acquired immunity along with their respective mechanism of action:

3.1 Innate Immunity

The immunity inherited by generation after generation, i.e., from parents to offspring, is called innate immunity. It means we get innate immunity by birth. Evolutionary innate immunity is very much **primitive** and it is estimated that every living creature including plants has some form of innate immunity to protect themselves from predators, particularly harmful agents/molecules and cells.

The other characteristics of innate immunity are as follows:

3.1.1 Non-Specific Antigenic

Instead of recognizing specific antigen(s) [**epitope(s**)], innate immunological cells recognize pathogen-associated molecular patterns **(PAMPs)** or damage-associated molecular patterns (**DAMPs**).

Table 1 Basic distinctions of innate and acquired immunity

Innate immunity	Acquired immunity
This primitive immunity is evolutionarily old	Most evolutionary developed immunity, only higher living creatures including humans can exhibit this
The first line of defense	The second line of defense
Non-specific	Highly specific
Quick response (within minutes)	Slow response (take days)
Does not form memory	Forms memory

3.1.2 Rapid Response

As soon as pathogens enter the human body, innate immunological cells recognize them, interact with them and gradually destroy them. Anticipated response time for innate immunity ranges within minutes.

3.1.3 No Memory

If and when, an exactly similar pathogen invades the body for the second successive time, the innate immunological cells cannot recognize the pathogen and on each occasion, react with pathogens by treating them as a new entity. In other words, the innate immunological cells do not exhibit any sort of memory against the pathogen and therefore cannot recognize if and when, an old pathogen previously infected the body.

3.2 Acquired Immunity

The immunity that a body or an organism attains in response to a specific pathogen (s)/antigen(s) is called acquired immunity. While growing as a fetus, passive transfer of acquired immunological molecules (e.g., IgG antibody) from the mother generally happens, but active acquired immunity only develops after the birth of a baby and exposure to the pathogens (antigens) from the environment.

The acquired immunity may have the following characteristics:

3.2.1 Highly Developed Immunity

Acquired immunity is evolutionarily the latest kind of immune response and only higher living creatures are capable of developing this.

3.2.2 Highly Specific

Each immunological cell reacts with a specific epitope (active site of an antigen).

3.2.3 Slow in Response

The pathogens are firstly **processed** by innate immunological cells before being **presented** to the acquired immunological cells. The whole process takes time. It is estimated that once a pathogen enters a body at least 7–8 days are needed for **antibody (Ab)** generation by the B cells or to **produce target or effector cells** by T cells against the processed antigen-MHCI/antigen-MHCII.

3.2.4 Forms Memory

The acquired immunological cells give rise to memory cells in the first exposure to the pathogen/antigen. If and when the same type of pathogen enters the same body again, the acquired immunological cells quickly recognize it, subsequently undergoing proliferation, differentiation, and rapid destruction/elimination of the pathogen.

3.3 Mechanism of Innate Immunity

Innate immunity is mediated* via *three mechanisms:

Physical Barrier
Chemical Barriers
Cells

Ahead is a brief discussion about them:

3.3.1 Physical Barrier

The physical barrier is mediated by the following:

- Skin
- Mucous membrane

The respiratory and gastrointestinal tracts are lined by mucus membrane, which entraps the microorganism. The respiratory tract is also covered by cilia, propelling the mucus entrapped microorganisms.

3.3.2 Chemical Barriers

The chemical barrier is mediated by the following:

- pH
- Lipids
- Enzymes, etc.

The sweat secreted by the sweat glands is acidic. The sweat also contains hydrolytic enzymes such as lysozymes. Both acidic pH and lysozymes work together to inhibit microbial growth on the surface of the skin. Therefore, skin acts as an important protective layer of the body, and under normal physiological conditions, the microorganisms including pathogens cannot grow unless there are injuries or persistence of skin lesions.

3.4 Cells Involved in Innate Immunity

The following cells are involved in the development of innate immune response:

Monocytes and Macrophages
Dendritic Cells
Natural Killer Cells
Basophils.
Polymorphonuclear Leukocytes (Neutrophils)
Eosinophils

NB: These innate immunological cells produce various molecules like cytokines, chemokines, interferons, pattern recognition molecules, reactive oxygen species (ROS), reactive nitrogen species (RNS),* etc. *that protect against pathogenic microorganisms.

Here is a brief discussion about the major immunoreactive molecules:

3.4.1 Cytokines

Low molecular weight (MW), soluble, secretory glycoproteins released by one cell population which act on another cell. For example, Interleukin 1 beta (**1L-1β**).

3.4.2 Chemokines

These are small positively charged secretor proteins facilitating the migration of various leukocytes. Example: Macrophage Chemotactic Protein 1 (**MKP-1**).

3.4.3 Interferons

A type of cytokine that kills the viruses. For example, Interferon-gamma (**IFN-γ**).

4 Pattern Recognition Molecule(s)

These are the molecules prevailing on the surface of phagocytic cells (e.g., macrophages) and interact with pathogen-associated molecular patterns (**PAMP**). Examples of pattern recognition molecules are Toll-like receptors (**TLRs**) which function as signaling molecules.

Besides the above molecules, certain **plasma proteins** are also involved in eliminating microorganisms. Examples of these plasma proteins are complement proteins and acute-phase proteins.

Here is a very brief discussion about them:

4.1 Complement System

- Complements are a heat-labile component of blood plasma proteins (~**50 plasma proteins**) that augment the phagocytosis of microorganisms.
- The complement proteins get activated step by step in a signaling cascading regime and finally aid in the elimination of pathogenic microorganisms by three distant pathways. These pathways are as follows: (1) **Classical pathway**, (2) m**annose-binding lectin pathway**, and (3) a**lternative pathway.**
- While complement systems are recognized as part of innate immunity, comprising of antibodies which are recognized as constituents of acquired immunity, essential for the elimination of microorganisms in the classical complement pathway.

4.2 Opsonization

Opsonization is recognized as one of the defense mechanisms of a mammalian body in which the disease-causing pathogens are coated by an Ab (such as IgG). Later on, the antigen-presenting cells (**APCs**) such as macrophages recognize these Ab-coated pathogens and engulf them by the process of **phagocytosis.** The complement system, thereby, plays a decisive role in opsonization.

4.3 Chemotaxis

Chemotaxis refers to the movement of cells, including **phagocytic cells** such as **macrophages** and **neutrophils** in response to various chemicals or **chemokines** such as macrophage chemotactic protein 1 **(MCP-1)**. This phenomenon allows the phagocytic cells to move toward the pathogens and therefore initiates the process of engulfment and destruction. Thus, bringing immune cells to the proximity of identified pathogens improves the likelihood of threat destruction and subsequent treatment of the infection.

4.4 Cell Lysis

The word **lysis** denotes the breakdown of a foreign cell or pathogen via predominantly targeting the plasma membrane proteins by the complement-mediated actions of phagocytic cells. As a consequence, the pathogenic cells undergoing lysis cease the capacity to proliferate and infect other organs.

4.5 Agglutination

Each antigen is characterized by an active site, called an **epitope**, and the counterpart of this for an Ab is referred to as a **paratope.** While one Ab has two paratopes, an antigen may have a single or multiple epitope(s). The strength of an epitope that binds a paratope is called **affinity**. The collective strength of binding of all epitopes with the paratopes is called **avidity**. ***The non-covalent bonding such as hydrophobic, ionic, and van der Waals forces are responsible for epitope-paratope interaction.*** **Agglutination** is the process of interaction of a multivalent antigen having several epitopes (or several antigens having single epitopes) with several antibodies (paratopes), together resulting in a large insoluble antigen-antibody aggregate.

4.6 Acute-Phase Proteins

In response to microbial stimulation, the **liver produces** a heterogeneous group of blood plasma proteins, called acute-phase proteins. These proteins maximize the complement activation and microbial opsonization.

Examples:

- Mannose-binding proteins (**MBPs**)
- C-reactive proteins (**CRPs**)
- Cytokines (**1 L-1, IL-6, IL-8,** etc.)
- Serum amyloid protein A (**SAA**)

5 Acquired Immunity

Lymphocytes (B and T cells) are involved in acquired immunity. Acquired immunity is further sub-divided into **humoral** and **cell-mediated immunity**.

The following paragraphs briefly describe these distinct forms of immunity:

5.1 Humoral Immunity

The immunity mediated by the antibodies secreted from the antigen-challenged B lymphocytes (**plasma cells**), is called humoral immunity. Antibodies, therein, secreted in the blood are diffused to other body fluids such as **lymph** and Broncho-Alveolar-Lavage (**BAL**) fluid, etc. So, the immunological actions of antibodies mediated through these body fluids (blood/lymph/BAL/cerebrospinal fluid, etc.), in total, comprise humoral immunity. The humoral word originated from **humor**, meaning **body fluids**.

NB: The alphabet B comes from the Bursa Fabricius of birds and not from the bone marrow, from where mammalian B cells originate and mature.

5.1.1 Working Mechanism of Humoral Immunity

Acquired immunity is developed, as and when a pathogen enters a body. The acquired immunological molecules such as antibodies can be passively transferred from one person to another (e.g., IgG transfer from mother to fetus or IgA transfer from mother's milk to newborn baby, etc.) or actively produced within the human body, in response to a specific antigen. The secreted antibodies interact with the antigens via **opsonization, agglutination, precipitation,** or Ab-**dependent cell-mediated cytotoxicity (ADCC)**. ADCC is mediated by natural killer **(NK)** cells. While details are available in almost all immunology test books, here is a very brief discussion about the Ab and its molecular structure.

5.1.2 Antibody

- Antibodies are the antigen-binding glycoproteins, synthesized by antigen-challenged differentiated B lymphocytes (named plasma cells).
- When B lymphocytes are challenged with a specific antigen(s) or epitope (s) (an epitope is an active site of an antigen through which it binds with the active site of an Ab called paratope), these proliferate and differentiate into memory cells, expressing antigen-specific antibodies on their surface and plasma cells which secrete the antibodies into the blood plasma.

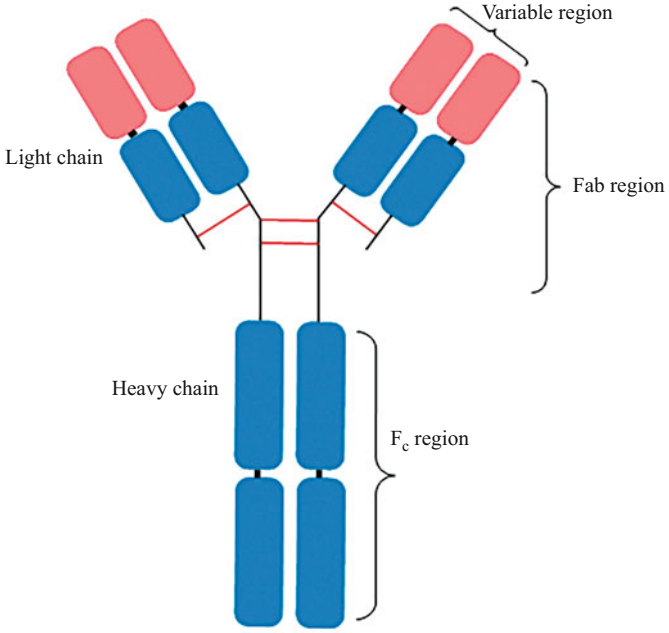

Fig. 1 Typical structure of an antibody, distinguished into heavy and light chains

- An Ab has a Y-shaped structure (Fig. 1).
- An Ab can bind two epitopes of antigen(s) simultaneously. So, in convention, an Ab is bivalent.
- An Ab has four polypeptide chains (Fig. 1).
- These chains consist of two light (called "L") and two heavy (called "H") chains. So, it is a heterodimer.
- In an Ab, both the light chains are identical.
- Similarly, both the heavy chains of an Ab are also identical.
- All mammalian species have two classes of light chains, namely kappa (κ) and lambda (λ).
- In a particular Ab, both the light chains are similar, i.e., both of them are either κ or λ. It should not be a mixture of one κ + one λ chain.
- There are five different types of heavy chains. They are **alpha (α), gamma (γ), delta (δ), eta (ε), and meu (μ)**. Based on the heavy chain distinctions, present antibodies can be divided into five different classes.

They are as follows:

Ig A: α (Alpha)
Ig G: γ (Gamma)
Ig D: δ (Delta)
Ig E: ε (Eta)
Ig M: μ (Meu)

Here is a brief discussion about them:

IgG: γ (Gamma)
- Made up of two heavy chains, called gamma.
- Most abundant serum Ab (~80%).
- Four subclasses (IgG1, IgG2, IgG3, IgG4).
- Present in monomer, small-sized and can passively pass through the placenta from mother to fetus.

IgM: μ (Mu)
- Made up of two heavy chains called Mu.
- The first Ab is produced by a mature B-cell surface (accounts for only 5–10% of the serum Ab).
- Also found on the surface of mature B cells together with IgD.
- The secreted IgM is pentameric, consisting of five IgM units linked by a polypeptide chain called the "**J chain/J peptide**" at the F_c region of the Ab.
- The largest Ab, and can bind ten epitopes of a multivalent antigen or ten different antigens having single epitopes and used in **agglutination reaction**.
- The pentameric IgM is highly efficient in "activating the complement system."

IgA: α (Alpha)
- Made up of two heavy chains called alpha.
- Comprise only 10–15% of the total serum Ab.
- ***A major Ab is present in the following secretions:***
  - Breast milk, colostrum
  - Saliva
  - Tears
  - Mucus of bronchial, genitourinary and digestive tracts.
- IgA has two subclasses: Ig A1 and Ig A2.
- IgA may form a dimer or tetramer with the J chain.

IgE: ε (Eta)
- Made up of two heavy chains called Eta.
- Through its Fc region, an IgE binds to the Fc receptor present on the mast cell membrane and basophil membrane.
- Activation of mast cells and basophil may lead to an **immediate hypersensitive reaction**.
- IgE-mediated degranulation is necessary for **anti-parasitic defense**.

IgD: δ (Delta)
- Made up of two heavy chains, called delta.
- Only 0.2% of the total Ab is in the serum.
- Both IgD and IgM are expressed on the same mature B-cell surface due to homology in the structure of the Fc region.
- Substantially aids in antigen recognition.
- Like IgM, may help in the **affinity maturation** of the B cells.

5.1.3 Molecular Structure of an Antibody

- Both light and heavy chains exhibit **variable regions** at their N-terminus, called V_L and V_H, respectively (Fig. 1).
- Both V_L and V_H constitute around 110 amino acid residues.
- Both light and heavy chains contain a constant region at the $–COO^-$ terminal region called C_L and C_H respectively.
- The C_H is three or four times larger (around 330 or 440 amino acids) than the C_L.
- In the variable region of both V_L and V_H, there are three **hypervariable regions**.
- These three hypervariable regions are called complementary determining regions (**CDRs**) since the epitopes of antigens complementarily bind with these regions.
- The remaining V_L and V_H domains exhibit fewer variations and are called **framework regions** or **FR**.
- While the light chain contains one variable and one constant domain, the heavy chain contains one variable and three constant regions.
- Both intra- and inter-chain disulfide bonds are responsible for holding the light and heavy chain together.

5.1.4 Deduction of Antibody Structure

Proteolytic enzymes are used to degrade an Ab molecule into definable fragments to facilitate the elucidation of an Ab structure.

Effects of Papain on Antibody

- Papain splits an Ab into three fragments of equal size.
- Two fragments, each has the separate capacity to bind to an antigen (an epitope). So, they are called Fragment antigen-binding or **FAB**. Each FAB is monovalent.
- The third fragment contains the $–COO^-$ terminus of the heavy chain. Since this fragment can be crystallized, it is called fragment crystallizable or $\mathbf{F_C}$.
- ***A typical F_C fragment has the following functions:***
  - Binds complements.
  - Secretes carbohydrate.
  - Dictates whether an Ab can cross the placenta or not.

Effects of Pepsin on Antibody

Treatment with pepsin digests a major portion of the F_c region in an antibody. Only one large fragment remains available, comprising two smaller **Fab fragments**, i.e., $\mathbf{F(ab)^2}$ linked together by a disulfide linkage. This is because $F(ab)^2$ binds two antigens simultaneously owing to its bivalent character.

5.1.5 Antigenic Determinants of Immunoglobulins

Biochemically, antibodies are **glycoproteins** in nature. So, an Ab produced by one person may act as an antigen in another person's body if both of them are not identical twins. The amino acid sequences at the variable and hypervariable regions of an Ab determine the type of epitope an Ab may bind. Thus, these variable and hypervariable regions of an Ab are also called **antigenic determinants**. Based on the antigenic determinants, antibodies are divided into three categories.

They are as follows:

Isotype
Allotype
Idiotype

Isotype

These antibodies are characterized by a similar type of amino acid sequence in the constant region of the heavy chain. Based on the isotype-heavy chain, the human antibodies are divided into five classes, namely, IgG, IgA, IgM, IgE, and IgD, as also discussed previously in this chapter. Each isotype is encoded by a separate constant region gene which is the same for all members of a species. Different species inherit distinct constant-region genes and therefore express dissimilar isotypes. Isotype differences substantiate from IgG versus IgA characteristics.

Allotypes

These antibodies are based on the genetic differences among individuals. Allotypes attribute their existence to the distinct allelic forms of the same gene. Example: IgG1 and IgG2 genetic variations in different individuals of the same species.

Idiotype

The idiotype determinants are generated by the conformations of heavy and light chain variable regions.

5.2 Cell-Mediated Immunity

- This type of immunity is mediated by T lymphocytes. Thymocytes, the T progenitor cells, are originated in the bone marrow and mature in the **thymus**. T cells are so-called because they are predominantly matured in the **thymus,** a small organ above the heart (discussed in Sect. 6.2.2).
- T cells recognize the antigens which are small peptides in nature.
- The T-cell receptor (**TCR**) present on the surface of T cells, along with another protein, **CD3 recognizes the peptide antigen**.
- The two types of T lymphocytes, **$CD4^+$ containing T helper (T_h)** and **$CD8^+$ containing T cytotoxic (T_C)**, are responsible for cell-mediated immunity.
- The $CD4^+$ and $CD8^+$ are also necessary for the recognition of peptide antigen for T_h and T_c, respectively.
- So, collectively, three molecules on the surface of T cells, i.e., TCR, CD^3, and CD^4/CD^8, are necessary for the recognition of the peptide antigen on the surface of either the **APCs** such as macrophages, dendritic cells, and B cells (for T_h cells) or **Target cells** (tumor/cancer cells, virus/protozoa infected mutated cells, etc.).
- However, peptide antigens are not presented to T_h/T_c alone but also to another molecule, that is called **major histocompatibility complexes** (**MHC**). There are two types of MHC: MHC class I and MHC class II.
- **While MHC I present antigens to Tc cells**; **MHC II presents them to T_h cells**.

NB: The third type of MHC, called MHC III although is well studied, but not relevant to antigen presentation.

Here is a very brief discussion of helper T cells and cytotoxic T-cell actions:

5.2.1 T Helper Actions

- As indicated before, the most important function of helper T-cells is to activate the B cells for their proliferation and differentiation into Ab-producing plasma cells and B-cell memory.
- Once activated by the APCs, the T_h cells release various cytokines such as interleukin-1 beta **(IL-1β)**.
- The released cytokines diffuse to the B cells and bind on the specific **IL-1β receptors** expressed on the surface of B cells, and subsequently activate B cells for their proliferation and differentiation.

  NB: Most notably the helping action of T_h to B cells gives it the nickname "T helper."
- The T_h may have several other functions including the following:
- Certain cytokines released by the T_h cells such as interleukin 10 **(IL-10)** suppress the immunological reactions and are therefore recognized as negative regulators.

5.2.2 Variants of $CD4^+$ Containing T Helper Cells

- The T_h cells are broadly divided into T_h1 and T_h2 cells. Recently, extensive studies discovered additional variants of $CD4^+$ containing T cells.
- These include the regulatory type 1 cells ($\mathbf{T_r1}$), T-helper 9 cells ($\mathbf{T_h9}$), T-helper 17 ($\mathbf{T_h17}$) cells, follicular helper T cell ($\mathbf{T_{fh}}$), and induced T-regulatory cells ($\mathbf{iT_{reg}}$).
- Analysis of the specific characteristics of these cells is facilitated by examining the release of cytokines and transcription factors from these cells followed by their epigenetic modifications (Fig. 2).

5.2.3 T-Cell Cytotoxic Actions

A $CD8^+$ T cell kills virus/parasite-infected and tumor or cancer cells by two mechanisms:

Perforin/Granzyme-Dependent Death
FASL/FAS-Dependent Death

Here is a brief discussion about these types of cell deaths mediated by T cytotoxic cells:

5.2.4 Perforin and Granzyme-Dependent Cell Death

T cytotoxic **(Tc)** T cells are also called cytotoxic T lymphocytes (**CTLs**).

Their mechanism of action is mediated by T_c or CTLs by releasing two distinct proteins, called perforins and granzymes.

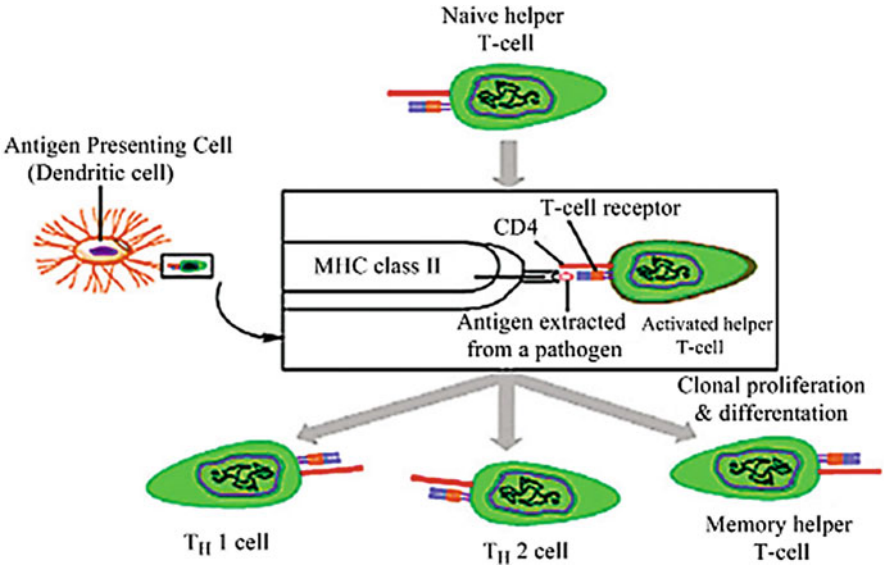

Fig. 2 Schematic depiction of a helper T-cell interception of an antigen-presenting cell (APC, through MHC class II complex) and its subsequent differentiation into cytotoxic and helper regimes

Following the interaction of T_c with the target cells (e.g., cancer/virus-infected cells), T_c releases perforins and granzymes. Several perforins have been discovered. The perforins released by the T_c cells diffuse to the target cells and make holes in the plasma membrane.

Through these holes the granzymes (granulated enzymes) enter the target cells, leading to **exocytosis**.

Biochemical analysis showed that the granzymes are **serine proteases**.

Granzymes are divided into **Granzyme A and Granzyme B**.

Ahead is a brief discussion about them:

Granzyme A

- The major function of Granzyme A is to cleave a subunit **mitochondrial complex 1** (the NADH dehydrogenase). This leads to the disrupted transfer of an electron from one complex to another, generating the reactive oxygen species (**ROS**), which eventually kills the target cells.
- **NB:** Mitochondrial electron transport chain consists of four complexes. They are C**omplex I, Complex II, Complex III, and Complex IV**. The electron carriers such as FAD and NAD transfer the electron from one complex to another leading to **oxidative phosphorylation** and ATP generation. Under normal physiological conditions, ~1% of the electrons may leak from complex III due to **single-electron reduction** leading to ROS generation.

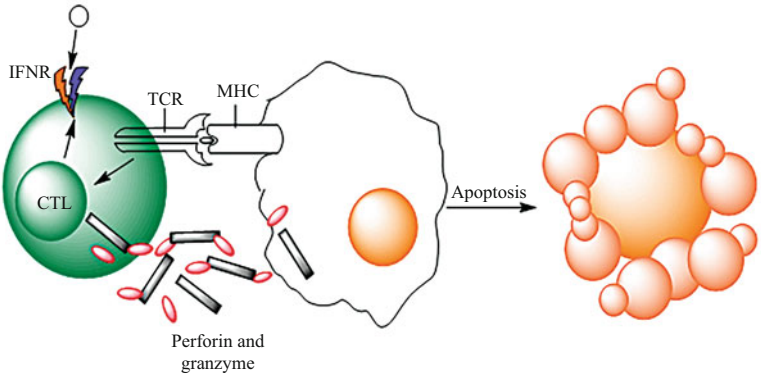

Fig. 3 Schematic representation of T-cell cytotoxic response and consequent cell death induction

Granzyme B

- Caspases are the enzymes that proteolytically cleaved various intracellular proteins, leading to the program cell death or apoptosis.
- Caspases are produced as inactive procaspases. During proapoptotic stimulation, various procaspases are cleaved into active caspases.
- Several experimental results showed that granzyme B cleaves the precursors of **caspases,** i.e., procaspases to active caspases.
- This, in turn, activates them to self-destruct the cells via apoptosis (Fig. 3).

5.2.5 FASL AND FAS-Dependent Death

- The full name of Fas is **FS-7-associated surface antigen**.
- Biochemically, it is a type II transmembrane protein and is included as a member of the TNF superfamily.
- The binding of T_c with the target cells leads to the high-level generation of FasL.
- The binding of FasL with the Fas receptors (FasR) of the target cells leads to their death via apoptosis, involving the sequential activations of ***Fas activating death domain (FADD)*** and caspases (Fig. 4).

6 Lymphoid Organs: Primary and Secondary

- Lymphoid organs are the sites where the lymphocytes originate and mature. These are the locations, where lymphocytes encounter antigens, getting proliferated and differentiated into various memory and active immunological cells (e.g., ***B cells proliferate and differentiate into plasma and memory cells, T_h cells proliferate and differentiate into memory and effector cells and T_c proliferate and differentiate into memory and target cells***).
- All the memory cells and active immunological cells are stored in the secondary lymphoid organs for future protection against pathogens/antigen attacks.

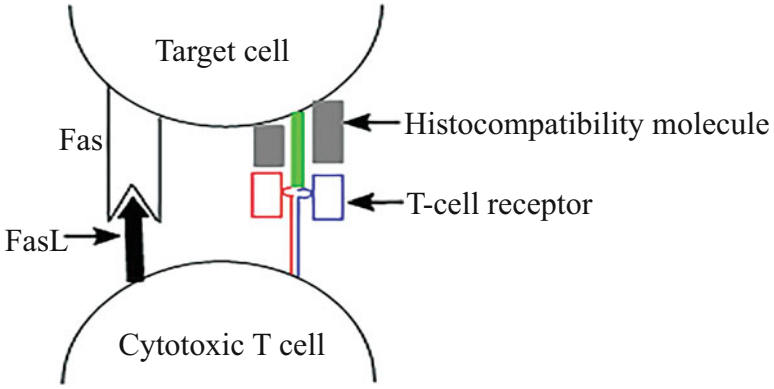

Fig. 4 Schematic depiction of the mechanism by which cytotoxic T cells induce their targets (e.g., virus-infected cells) toward suicide (apoptosis or programmed cell death)

- The organs and tissues of the immunological system can be classified into primary and secondary subtypes.

Below is a briefing on each of these:

6.1 Primary Lymphoid Organs

Primary lymphoid organs denote the organs from where all the lymphocytes (B/T lymphocytes **originated**. Thus, primary lymphatic organs provide an environment for hematopoietic stem **cells** to divide and mature into B and T cells. Two primary lymphatic organs are the **red bone marrow** and the **thymus gland.**

6.2 Secondary Lymphoid Organs

Secondary lymphoid organs and tissues are the regions where mature lymphocytes interact with an antigen and generate an immune response.

The major secondary lymphoid organs are as follows:

The Spleen
The Lymph Nodes
Mucosa-Associated Lymphoid Tissues (MALT)
Gut-Associated Lymphoid Tissues (GALT)

The major function of the secondary lymphoid organs is to further proliferate and differentiate the lymphocytes.

To discriminate between the self and non-self, the secondary lymphoid organs interact with the antigens (foreign cells) and eliminate them.

Storage of memory cells. The function of memory cells is to interact with the same type of antigens against which these cells are generated if, or as and when, these antigens again enter the same body (Marieb 2007; Crivellato et al. 2004).

Here is a brief discussion of primary and secondary lymphoid organs.

6.2.1 Red Bone Marrow

- **Red Bone Marrow** is a spongy substance found in the center of the **bones**.
- Following birth, human bone marrow manufactures the hematopoietic stem cells (**$CD34^+$**) from which all the blood cells including immunological cells such as lymphocytes (e.g., **$CD4^+$ B and $CD8^+$ T lymphocytes**) **originate**.
- However, in the **bird Bursa of Fabricius**, a dorsal out pocketing of the cloaca produces and matures B lymphocytes, leading to the origin of the terminology **"Bursa"** in the name.
- However, $CD8^+$ T cells do not mature in the bone marrow.
- Other innate immunological cells such as macrophages, eosinophils, neutrophils basophils, etc. originate and mature in the bone marrow.
- Unlike the thymus, the ***bone marrow does not exhibit atrophy at puberty*** and therefore, does not undergo a concomitant decrease in the B lymphocyte generation with age.

6.2.2 The Thymus Gland

- The thymus is a pyramid-shaped primary lymphoid organ, typically 4–6 cm long, 2.5–5 cm wide, and about 1 cm thick at birth. The bilobed thymus may weigh 40–50 g.
- Based on human developmental biology it is known that in humans, the thymus appears early in course of fetal development and continues to grow until puberty.
- After puberty, the thymus begins to shrink and one 80-year-old human retains only one-third of thymus size.
- Anatomically, the thymus is located just ***behind the sternum in the upper part of the chest (apical region of the heart)***. The histological section of the thymus illustrates that it comprises an outer, lymphocyte-rich cortex and an inner medulla.
- ***Since $CD4^+$ and $CD8^+$ lymphocytes are matured in the thymus, these cells are called T lymphocytes.***
- Of note, as indicated in the above paragraph, T lymphocytes originate in the bone marrow.
- The T-cell differentiation happens in the cortex of the Thymus (Fig. 5).

  NB: The T-cell production decreases with the age. This decrease in the production of T cells is related to the age-dependent decrement in thymus size.
- The T-cell receptors are originated in the thymus and the T cells exposed to Thymus dependent antigens.
- Finally, it is the thymus, where T cells become capable of distinguishing self and non-self MHC^+ antigen.

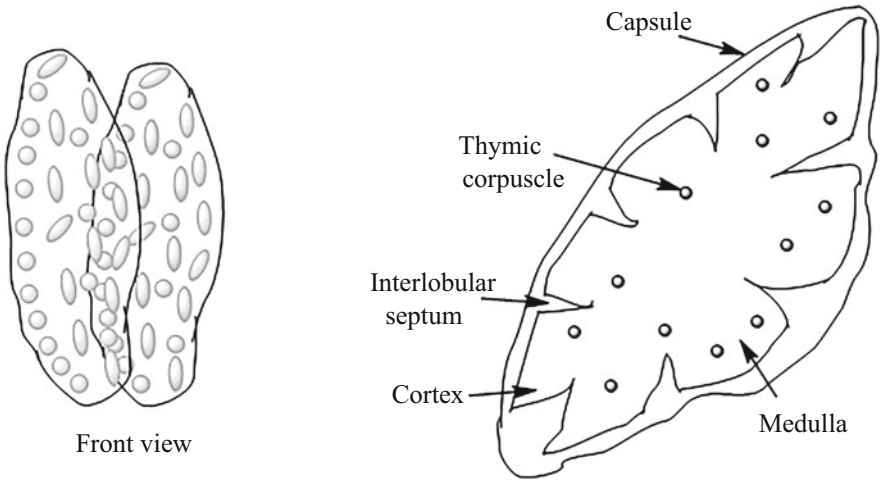

Fig. 5 Pictorial depiction of a thymus, front view, and internal morphology (right)

- Eventually, these cells tolerate self-cells containing self-MHC^{+} antigen and get acquainted to interact with and destroy the non-self-molecules **(*the whole process is called learning a lesion for self-tolerance*)**. For this learning, T lymphocytes have to undergo either positive or negative selection.

6.3 Secondary Lymphoid Organs

1. The B or T lymphocytes that are yet to interact with the antigens are called **Naïve Cells.** The naïve cells die within weeks if not encountered with antigen(s).
2. The secondary lymphoid organs are those where naive T and B lymphocytes interact with antigens before proliferating and differentiating.
3. The **T_h** cells proliferate and differentiate into **Memory** and **Effector cells.**
4. The **T_c** cells proliferate and differentiate into **Memory** and **Target cells.**
5. **B cells** proliferate and differentiate into Ab releasing **plasma** and **memory cells**.
6. While memory cells can prevent the likely diseased conditions by the same pathogens, the other cells such as target/effector/plasma cells help to effectively eliminate the antigens.
7. Innate immunological cells such as **macrophages** are also stored in the secondary lymphoid organs such as the spleen and get de-differentiated as per the need of the organs.
8. The most important secondary lymphoid organs are the **spleen, lymph nodes, Mucosa-, and Gut-Associated Lymphoid Organ**s (**MALT and GALT)**.

Ahead is a brief description of these secondary lymphoid organs:

6.3.1 The Spleen

- The spleen is one of the secondary lymphoid organs (~4 inches long), whose major function is the filtration of blood (not filtration of lymph). Figure 6 depicts the typical morphology of a spleen.
- Anatomically, the spleen is located on the left side of the abdomen, near the stomach.
- Histologically, the spleen consists of two tissues, **the Red pulp, and the white pulp.**
- While the red pulp contains the innate immunological cells, called **macrophages** (also called **splenic macrophages**), the white pulp contains **B** and **T lymphocytes**.
- Further, histological analysis revealed that T cells congregate around the tiny arterioles which enter the spleen. On the other hand, the B cells are located in regions called **germinal centers.**
- It is this germinal center, where the B lymphocytes expose to the antigens leading to their proliferation and differentiation to Ab-producing **plasma cells** and **memory B cells**.
- While a single plasma cell releases a few 1000 similar antibodies into the circulation, the memory cells are stored in the spleen for years and wait for encountering the same antigen against which these are programmed.
- If or, as and when the same type of antigen consecutively enters the same body or spleen, the memory cells rapidly recognize the antigen, effectively proliferating and differentiating to eliminate the antigen.

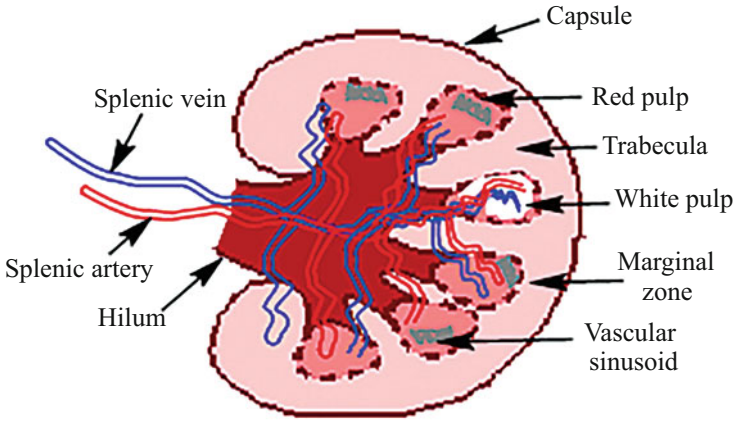

Fig. 6 Schematic representation of the ***major immunosurveillance unit*** of the physiological system, the spleen

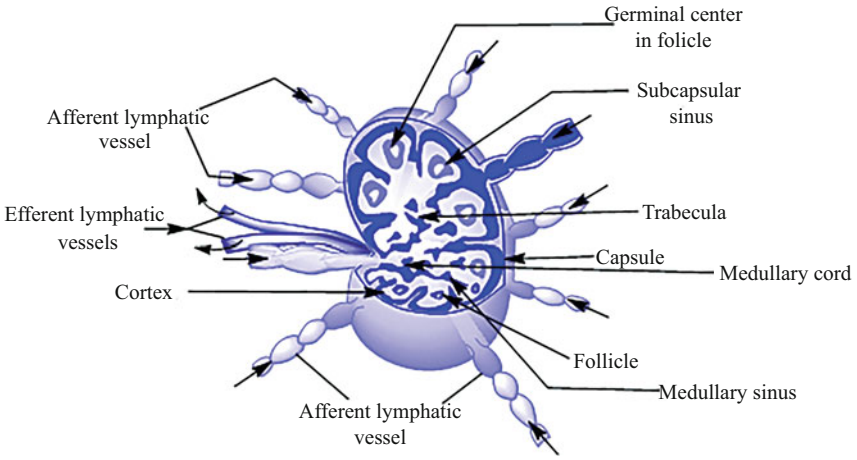

Fig. 7 Internal morphology of a lymph node, distinguished into multiple sub-compartments

6.3.2 The Lymph Nodes

- The lymph nodes (**LNs**) are synonymous with **lymph glands**.
- These are small bean-shaped structures found throughout the human body and the main function of a lymph node is to **filter lymph** (Fig. 7).
- However, these structures are prevalent in the areas around the **armpits (axillary nodes), groin (inguinal nodes), neck (cervical nodes),** and **knees (popliteal nodes).**
- Cross-sectional histological studies showed that a lymph node consists of lobules or tubules.
- Further, each lobule consists of three parts. They are **a region of the cortex with combined follicle B cells (also called the germinal center), a paracortex of T cells, and a part of the nodule in the medulla**.
- The lymphocytes enter lymph nodes through specialized blood vessels called as **high endothelial venules**.
- Regarding the mechanism of elimination of pathogens/antigens, the antigen-carrying lymph drains into the lymph node through afferent (incoming) lymphatic vessels and percolates through the lymph node.
- Now, lymph-carrying antigens come in contact with lymphocytes and activate them.
- In the next step, the activated lymphocytes, carried in the lymph, exit the node through the efferent (outgoing) vessels before eventually entering the blood-stream and getting distributed throughout the body.

6.3.3 The Mucosa-Associated Lymphoid Tissues/Gut-Associated Lymphoid Tissues

- Mucosal surfaces of any organ may be protected from the various germs by immunological cells including B and T lymphocytes stored in these places.

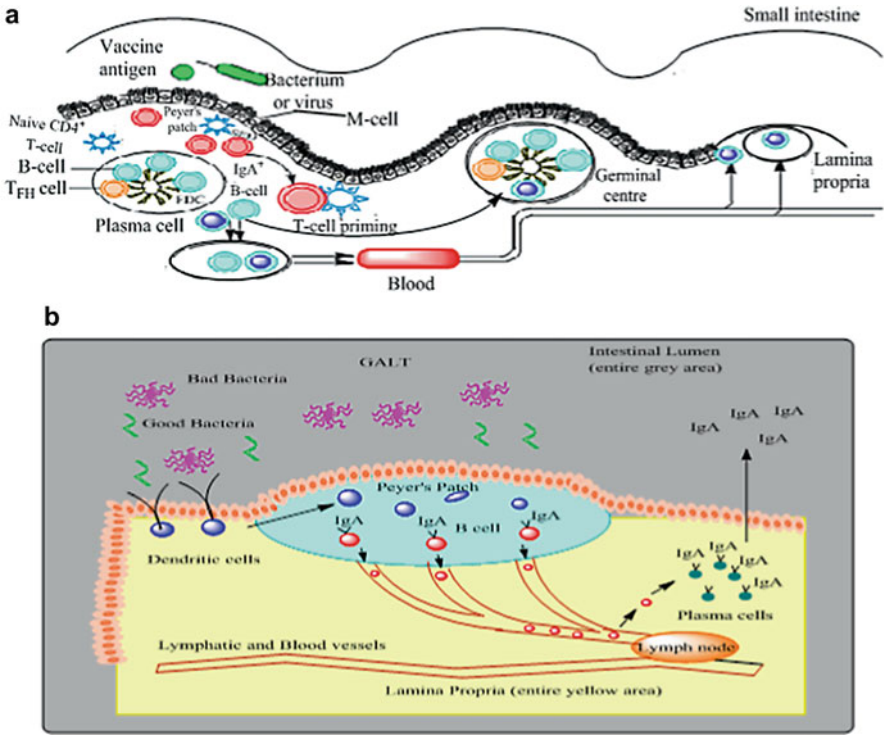

Fig. 8 Schematic representations of (**a**) Mucosa-Associated Lymphoid Tissue (MALT) and (**b**) Gut-Associated Lymphoid Tissue (GALT)

- However, it is necessary to take special attention to the immunological protection of the tissues, organs, and systems that are directly exposed to the external environment and are likely to be invaded by various germs or pathogens.

  The tissues/organs associated with these systems are as follows:

  The Digestive Tract
  The Respiratory Tract
  The Genitourinary Tract
- Collectively, the mucosa of these tissues/organs is called the ***mucosa-associated Lymphoid Tissues (MALT)*** (Fig. 8a).
- The respiratory epithelium-associated lymphoid aggregates are sometimes referred to as the **bronchial-associated lymphoid tissue (BALT)**.
- On the other hand, the intestinal epithelium-associated lymphoid aggregates are sometimes referred to as the **gut-associated lymphoid tissue** (**GALT**) (Fig. 8b).
- **Peyer's patches** are the cells present in the mucosa and submucosa of the small intestine.

- The lymphocytes in the GALT are located within the epithelial layer, scattered through the **lamina propria,** and clustered as organized collections in the lamina propria. The latter includes the **tonsils, adenoids,** and **appendix**.
- Experimental results identified that $CD8^+$ containing T_c cells infected the gut epithelial cells. Further studies showed that 10% of these cells express the γ/δ form of the TCR.
- On the other hand, the intestinal lamina propria contains a mixed population of cells including activated $CD4^+$ T cells.

7 Cells Involved in Immunity: Innate and Acquired Immunological Cells

- **Blood** is the source of all immunological cells. The process of synthesis of blood is called **hematopoiesis**.
- **In humans, the hematopoietic stem cells in the red bone marrow are the source of all blood cells** (Table 2).
- Hematopoietic stem cells (**$CD34^+$**) were firstly discovered in 1961.
- Hematopoietic stem cells yield to two different blood cell lineages, namely, **myeloid and lymphoid progenitor cells**.
- The myeloid progenitor cells proliferate and differentiate (called **myelopoiesis**) into monocytes/macrophages, eosinophils, neutrophils, basophils, mast cells, platelets, and RBCs. Except for platelets and RBCs, all these cells are involved in **innate immunity** and used as **accessory cells for adaptive immunity**.
- The lymphoid progenitor cells proliferate and differentiate (called **lymphopoiesis**) into lymphocytes (**B lymphocytes and T lymphocytes**), dendritic cells, and natural killer (NK) cells. While dendritic and NK cells are involved in innate immunity, B and T lymphocytes **are involved in acquired or adaptive immunity**.
- The B and T lymphocytes are involved in humoral and cell-mediated immunity, respectively.
- The process of RBC synthesis from myeloid progenitor cells is called **erythropoiesis**.

Table 2 Origin of blood cell formation

Developmental stages of fetus	Place of origin of blood cells
First-week embryo	Yolk sac
Third month	Fetal liver
Third to seventh months	Fetal liver and spleen
Seventh to tenth months	Fetal bone marrow (predominantly), spleen, and liver (a little), bone marrow starts producing a little number of blood cells
After birth	Bone marrow only/bursa of fabricius (birds)

NB: B and T lymphocytes that do not interact with the antigen are called Naïve Cells. These cells will die within days if not encountered with the antigens. However, on encountering the antigens, these cells proliferate and differentiate into memory cells that are stored in the secondary lymphoid organs and have a lifespan of years.

7.1 Source Organs of Immune Cells

- T lymphocytes are found in lymph nodes (LN), spleen, and thymus.
- B lymphocytes are found in LN and spleen.
- Natural killer (NK) cells are found in LN and spleen.
- NKT cells are found in the thymus, spleen, and liver.
- Monocytes are found in the blood.
- Macrophages are found in LN, spleen, liver, lung, brain, and bone marrow.
- Dendritic cells are found in LN and spleen.
- Neutrophils, eosinophils, and basophils are found in blood, spleen, lung, and liver.
- Platelets are found in the blood.

Ahead is the brief presentation of all the innate and acquired immunological cells.

7.2 The Monocytes and Macrophages

- Innate immunological cells, i.e., monocytes get differentiated into macrophages.
- Through circulation, macrophages enter various organs and gain the capacity to become adherent. Due to the expression of tissue/organ-specific surface marker proteins, the de-differentiated macrophages are given specific names (Table 3).
- Macrophages engulf the pathogens via phagocytosis.
- Macrophages produce various molecules such as cytokines, chemokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), etc.
- These molecules kill the phagocytosed pathogens within the macrophages.
- Macrophages then process the engulfed pathogens.

Table 3 De-differentiation of macrophages into various cell types

De-differentiated macrophages	Organ present
Microglial cells	Central nervous system (CNS)
Alveolar macrophages	Lung
Osteoclast	Bone
Splenic macrophages	The white pulp of the spleen
Peritoneal macrophages	Peritoneal fluid
Kupffer cells	Liver
Mesangial cells	Kidney

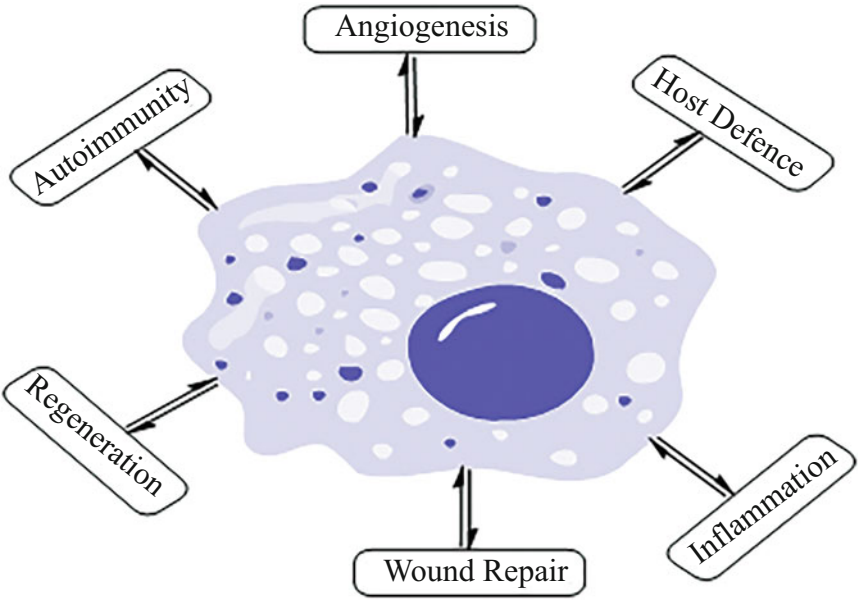

Fig. 9 Schematic representation of a macrophage workable functioning

- The processed pathogens bind with MHC II and are expressed on the macrophage surface. Finally, the antigen-MHC II complex is presented to acquire immunological cells such as B cells. So, macrophages act as one of the primary APCs (Fig. 9).

  NB: B cells and dendritic cells are other primary APCs. Based on the type of cytokines and other mediators release macrophages are divided into M1 and M2 types. While M1 macrophages protect against pathogens and inhibit cell proliferation, the M2 macrophages show immunosuppressive function due to the production of various immunosuppressive cytokines (e.g., IL-10). These kinds of macrophages also help the tumor and cancer cells to survive and proliferate.

7.3 The Eosinophils

- A type of WBC (granulocytes), consisting of a bilobed nucleus (Fig. 10).
- These cells stain with **hematoxylin** (nucleus) **acidic stain eosin** (cytoplasm).
- These protect against **protozoa and helminth parasite** infection by releasing cationic peptides and reactive oxygen species (ROS).

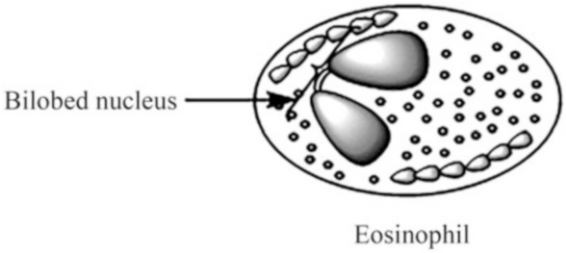

Fig. 10 Pictorial representation of an eosinophil structure

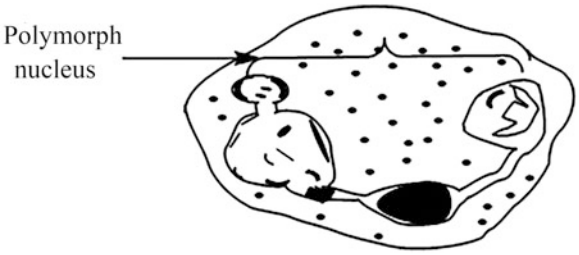

Fig. 11 Depiction of a neutrophil morphology

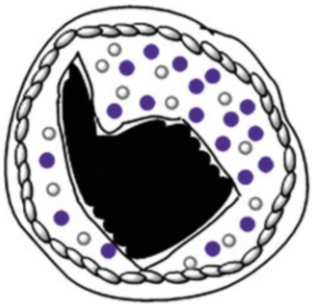

Fig. 12 Depiction of basophil morphology

7.4 The Neutrophils

- The most abundant 60–70% of granulocytes are within a polymorph nucleus (Fig. 11).
- The cell cytoplasm is stained with a **neutral stain**.
- Like macrophages, neutrophils are active phagocytic cells.

7.5 The Basophils

- These cells are large granulocytes with a single or bilobed nucleus (Fig. 12).
- Their cell cytoplasm stains with the **basic stain hematoxylin**.
- Basophils release histamines, prostaglandins, serotonin, and leukotrienes.

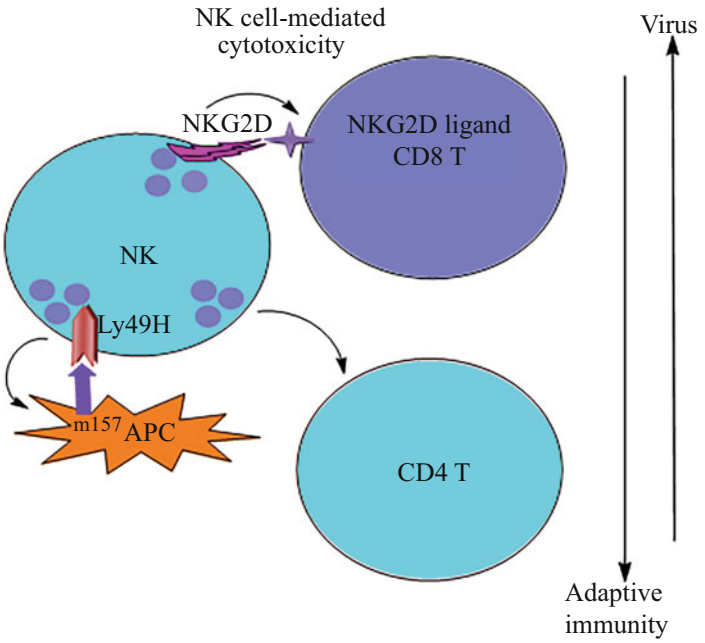

Fig. 13 NK cell-mediated cytotoxicity for regulating immune responses to viral infections. Noticed NK cell-mediated immuno-regulatory effects are accompanied by reduced resistance and persistence of viral infections

- Basophils possess the ***receptor for the Ab IgE, which is involved in type-1 hypersensitive reactions***.

7.6 The Natural Killer Cells

- Large granular lymphocyte-like cells (Fig. 13).
- These cells kill the tumor/virus-infected cells via cytotoxic action.
- While the mechanism of action of natural killer (NK) and cytotoxic (T_c) cells is quite similar (perforin-granzyme dependent death), the NK cell's action predominantly happens via an innate immune mechanism in contrast to adaptive immune sensitivity for T_c.
- Here are the basic differences between NK cells and T_c (Table 4).

7.7 The Mast Cells

- Mast cells were discovered by ***Paul Ehrlich*** in 1877–1878.
- Another name for a mast cell is **mastocyte** or a **labrocyte.**
- While mast cells look like basophils, these are two types of cells with completely different lineages (Fig. 14).

Table 4 Differences between natural killer and cytotoxic T cells

Natural killer cells	T cytotoxic cells
Directly interact with unprocessed antigens	Interact with processed antigens (a small peptide as antigen)
Do not need MHC for an activity or to present antigen(s)	Interact with only MHC-I + processed antigens (peptide)
Do not form the memory cells and survive for a short duration (~21 days)	Form the memory cells, can be stored in the secondary lymphoid organs and survive for years
Through CD16 binds with IgG-bound target cells (e.g., cancer cells) and involve in Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)	Are not involved in ADCC
Recognized as part of the innate immunological system	Recognized as part of the acquired immunological system

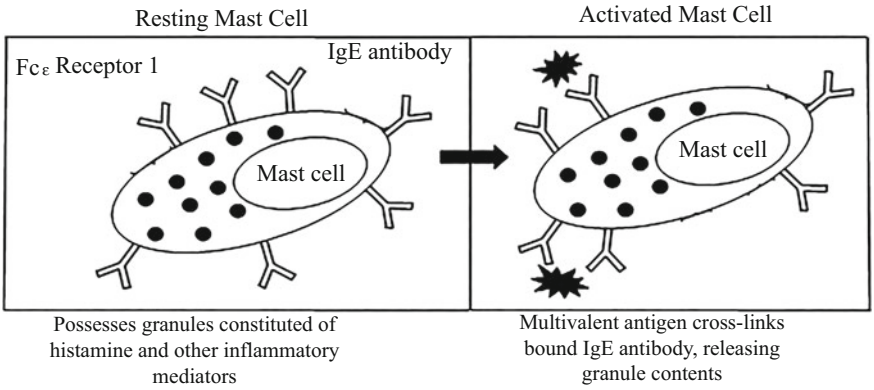

Fig. 14 Resting and activated stage distinct functions of a mast cell

- These are myeloid-derived cells that are rich in **histamine** and **heparin-rich granules**.
- Other molecules that mast cells release are **proteases, prostaglandin D2, leukotrienes, and a variety of cytokines.**
- Studied for their involvement in **type 1 hypersensitive reaction** mediated by **IgE Ab**. Also associated with the **anaphylactic reaction**.
- May have a role in immune defense against **parasites.**

8 The Lymphocytes

As described previously that although in humans, B cells originated and matured in the bone marrow, the B alphabet was introduced in the name because of its first notice in ***Bursa Fabricius*** of birds. Table 5 comprises the molecules which a B cell can express on its surface.

Table 5 Molecules expressed on the surface of B cells with their functions

Molecules expressed on B-cell surface	Functions
Antibodies. Initially, IgM and IgD are expressed. After interacting with specific antigens may class be switched to IgG/IgA or IgE	Interact with antigen
B-cell receptors (Igα, Igβ)	Interact with antigen
B-cell co-receptor (CD19, CD81 (also known as TAPA-1) CD21 (CR2). CD^{19} is recognized as a specific identifying marker of B cells	Interact with antigen
Class II MHC molecules	Permit the B cell to function as an antigen-presenting cell (APC)
CRI (CD 35) and CR2 (CD 21)	Receptor for certain complements
FcrRII (CD 32)	IgG binding
B7-1 (CD 80) and B7-2 (CD 86)	The B7-1 and B7-2 act as co-stimulatory molecules. Build with CD28 of Th and cytotoxic T lymphocyte antigen 4 (CTLA-4) of Tc respectively.
CD 40	Survival of B cells
CD 45	B-cell marker (unlike Ab not unique, only for B cells)

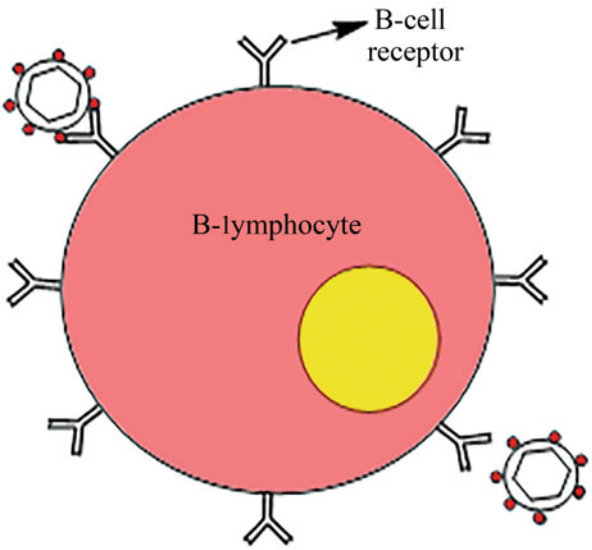

Fig. 15 Immunological response exercised through B-lymphocyte, distinguished based on interacting through B-cell receptor

Figure 15 (above) describes the B-cell receptor-driven explicit immunological response exercised by a B-lymphocyte.

8.1 B-Lymphocyte Functions

B lymphocytes have the following functions:

8.1.1 Antibody Production

Antibody produced and secreted by the B lymphocytes may have the following functions:

Opsonization

Coating of foreign particles by the Ab, followed by recognition and building of coated Ab by the phagocytic cells. The phagocytic cells phagocytose the Ab-coated foreign materials.

Neutralization

Antibodies can neutralize the attacking toxins or virus particles.

Precipitation

This refers to the interaction of a soluble antigen with a soluble Ab to form an insoluble complex (Fig. 16).

Agglutination

It is an interaction of several antibodies with a multivalent antigen having several **epitopes** or several single epitopes, combining antigen to a single Ab (e.g., pentavalent IgM) to form a large insoluble antigen-antibody aggregate, termed agglutinate (Fig. 17).

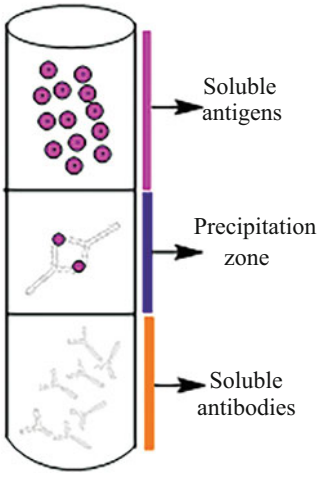

Fig. 16 General process of a precipitation reaction, separating soluble antigen using a soluble Ab

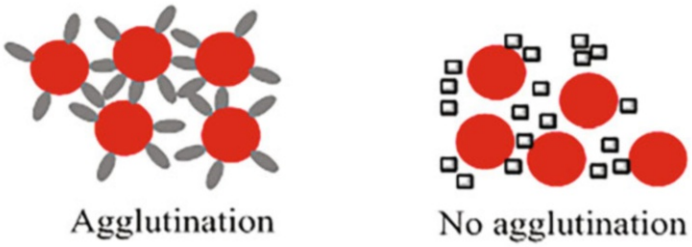

Fig. 17 General process of agglutination, wherein multiple antibodies interact with manifold single epitopes on a multivalent antigen

Table 6 Summarized properties of B cells

Origin	Bone marrow
Maturation	Bone marrow (*Bursa of Fabricius* in birds)
Expression of Ag receptors	Bone marrow
Differentiation	Lymphoid tissue
Surface immunoglobulin	Present
Immunity	Humoral
Distribution	Spleen, lymph node, bone marrow, and other lymphoid tissue
Secretory product	Ab
Complement receptor	Present

8.1.2 Complement Activation

B cells have complement receptors (CR1, CR2) on their surface, aiding in the binding of complements and their subsequent activation.

8.1.3 Antibody-Dependent Cell-Mediated Cytotoxicity

- Infected/malignant cells may be coated with antibodies (IgG).
- NK cells express CD16 receptors on their surface.
- CD16 recognizes the Ab-coated cells, bind with the F_c region of an antibody, and thereafter NK cells destroy the Ab-coated cells.
- This is called **antibody-dependent cell-mediated cytotoxicity (ADCC)**.

8.1.4 Antigen Presentation

B cells act as **APCs**. **Antigen-MHC II complex** prevailing on the B-cell surface is presented to T cells.

Table 6 summarizes the prominent traits of the B cells.

8.1.5 B-Cell Activation

Activation of B cells requires antigens. The following two different pathways are typically involved.

T_h Cell-Dependent Activation of B Cells
T_h Cell-Independent Activation of B Cells

T_h Cell-Dependent Activation of B Cells

T_h and B cells cooperate in the development of thymus-dependent antigens. B cells act as one of the primary APCs. Class II MHC presents a small peptide antigen (**exogenous antigens**) to T_h cells. Thymus-dependent antigen requires direct contact with T_h cells, not merely the exposure to T_h-derived cytokine. The following possibilities could be noticed as a response to thymus-dependent antigens:

Synthesis of Memory Possessing B Cell.
Affinity Maturation.
Class Switching.

T_h Cell-Independent Activation of B Cells

T_h cell-independent B-cell activation involves the direct interaction of antigen with B cell without thymus involvement (Fig. 18). These antigens are large polymorphic molecules with multiple repeating antigenic determinants. Example, LPS from bacterial cell wall or capsular protein of *H. Influenza.* DNA/RNA derived from pathogens or some other molecules also act as antigens.

8.1.6 Clonal Selection

- Clonal selection theory was proposed by Nobel laureate ***Sir Macfarlane Burnet*** who explained the response of the adaptive immune system to millions of different antigens in a highly specific manner.
- Clonal selection theory postulates that the adaptive immune system functions on a ***ready-made*** principle rather than the ***made-to-order one***.
- An animal/human first ***randomly generates a vast diversity of lymphocytes.***
- Each of the lymphocytes is ***committed*** to a ***particular antigen.***
- So, each lymphocyte acts as a ***clone***.

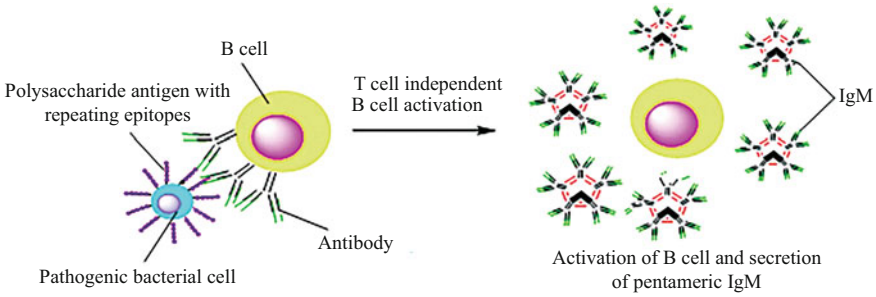

Fig. 18 T_h cell-independent B-cell activation, characterized via IgM expression

- When encountered by a particular antigen in the peripheral lymphoid organ, the particular lymphocyte proliferates and differentiates into a vast number of similar lymphocytes.

8.2 T Lymphocyte

- ***Originates in the bone marrow and matures in the thymus.***
- Two types: ***T helper (T_h) and T cytotoxic (T_C)***.
- T_h carries $CD4^+$ glycoprotein on its surface.
- T_h divided into T_h1 and T_h2.
- T_h1 secrets IL-2, IFNγ, and TNFβ: responsible for delayed hypersensitivity and activation of cytotoxic T cells, NK cells, and macrophages.
- T_h2 synthesizes IL-4, IL-5, IL-10, and IL-13, enabling the B-cell activation as well as cell-mediated immunity.
- T_C expresses the $CD8^+$ glycoprotein on its surface.
- Both T_h and T_c carry a CD3 complex on their surface.
- CD3: Five polypeptide chains forming five dimers. CD3 acts as a chaperone to synthesize and transport TCR.
- T_c is involved in the destruction of virus-infected cells, tumors, and cancer cells, as described previously.

  NB: CD3 is exclusively expressed on the T-cell surface. So, it is a T-cell molecular marker.
- T-cell receptor (TCR): Heterodimer consists of α and β chains connected by a disulfide bond. They are called αβ TCR. A major type is the target processed peptides + MHC 1.
- **The γδ TCR:** Consists of the γ and δ chains. γδ TCR is very uncommon, being neither recognized nor presented in the context of MHC molecules.
- TCR interacts with + MHC 1 peptide. Ig interacts with carbohydrates, DNA, lipids, and proteins. Table 7 distinguishes the αβ and γδ T cells, based on their characteristic T-cell receptors.
- Naïve or effector T cells never secrete TCR.
- The activated T cells secrete cytokines.
- **CD28:** A co-receptor for T cells that interacts with the B7 family of molecules present on B cells and other antigen-presenting cells.
- **CD45:** T cells contain CD45, a signal transduction molecule.

  NB: TCR is monovalent and Ig is bivalent.

Table 7 Comparison of α β and γδ T cells

Features	αβ T cells	γδ T cells
CD^4	Present	Absent
CD^8	Present	Absent
MHC restriction	$CD4^+$ MHC class II $CD8^+$ MHC class I	No MHC restriction
Ligands	Peptide + MHC	Phospholipid antigen

8.2.1 Functions of T Lymphocytes

- A mouse lacking a thymus is called a ***"nude mouse."***
- *A child lacking a thymus is known to suffer from* ***DiGeorge*** **Syndrome**.
- A T cell that does not encounter an antigen is called a ***"naive T cell."***
- A T cell can get activated by + MHC peptide of APC as well as by B7 production, subsequently interacting with CD28.
- Once activated, the naïve T cells enlarge into blast cells.
- The ***blast cells*** proliferate and differentiate into **memory** and **effector cells**.

The effector cells have the following functions:

Secretion of cytokines.
B-cell activation.
Cytotoxicity-induced killing.

8.2.2 Function of T Helper Type 1 Cells

T_h1 is responsible for delayed hypersensitive reaction and the activation of the cytotoxic T lymphocyte, NK cells, and macrophages. It mediates the prominent inflammatory reactions.

8.2.3 Function of T Helper Type 2 Cells

Acts as B-cell helper.

8.2.4 Functions of Cytotoxic T Lymphocytes

The cytotoxic T lymphocytes interact and kill the following cells:

- Virus-infected cells
- Bacteria infected cells
- Tumor/cancer cells
- Transplanted cells

The cell killed by T_c is called the target cell. The **T_c–target cell interaction** results in the death of the target cells. T_c also expresses IFNγ which regulates viral and bacterial infections. TNFβ aids in killing the cells. The mechanism of T_c action has already been discussed in previous pages. A summary of the T-cell properties is presented in the following table (Table 8).

Table 8 Salient properties of T cells

Origin	Bone marrow
Maturation	Thymus
Expression of Ag receptor	Thymus
Differentiation	Lymphoid tissue
Surface Ig	Absent
Immunity	Cell-mediated + T_h helps in humoral immunity
Secretory product	Cytokines
Receptor	TCR on membrane

Ag antigen, *TCR* T-cell receptor

8.3 T-Cell Maturation

- The T-cell precursor originates in the bone marrow and moves to the thymus for maturation.
- In the thymus, the pro-T cells lack $CD4^+$ and $CD8^+$ co-receptors. So, they are called **double negative cells**.
- In the thymus, the double negative pro-T cells are converted to **double-positive** ($CD4^+$, $CD8^+$) pre-T-cells. These double-positive cells also express TCR.
- The double-positive pre-T cells undergo multiple changes through a process called ***thymic selection***.
- If TCR from the double-positive cell recognizes and interacts with the self MHC and self-peptide expressed on the **epithelial cell surface** of the **thymus cortex,** they undergo **positive selection**.
- The positive selection helps the T cells to screen and identify the ***self-MHC***. For this particular call, all other MHC molecules are referred to as **non-self**.
- The double-positive cells which learn the lesson of non-self-MHC, never interact with self-MHC-containing cells in their life span. These cells only interact with the non-self-MHC, a phenomenon termed a **self-MHC restriction**.
- The double-positive self-MHC restricted cells may interact either **very strongly** or **weakly** with self-MHC.
- The very strong interaction with self MHC may cause severe consequences including the destruction of thymic cells. So, the **negative selection** takes place.
- **Dendritic cells** expressing self-MHC on their surface take charge of negative selection. A T cell that expressed TCR, reacts too strongly with the dendritic cell MHC and is selected to **death by apoptosis**. Only those T-cells survive which do not interact too strongly. Thus, negative selection removes the T-cell expressing TCRs with high reactivity to self-components, manifesting **self-tolerance**.
- Finally, the double-positive cells express either $CD4^+$ or $CD8^+$ by an unknown mechanism, are released from the thymus, and move to the secondary lymphoid organs.
- Figure 19 (ahead) summarizes the step-by-step procedure of T-cell maturation, via recognition of self and non-self MHC.

9 Steps to Collect Immunological Cells

The collection of immunological cells requires knowledge of the following steps:

1. Animals used in immunological research
2. Euthanasia of the animals
3. Organ harvesting
4. Isolation of cells from the collected organs

Ahead is a brief discussion about them:

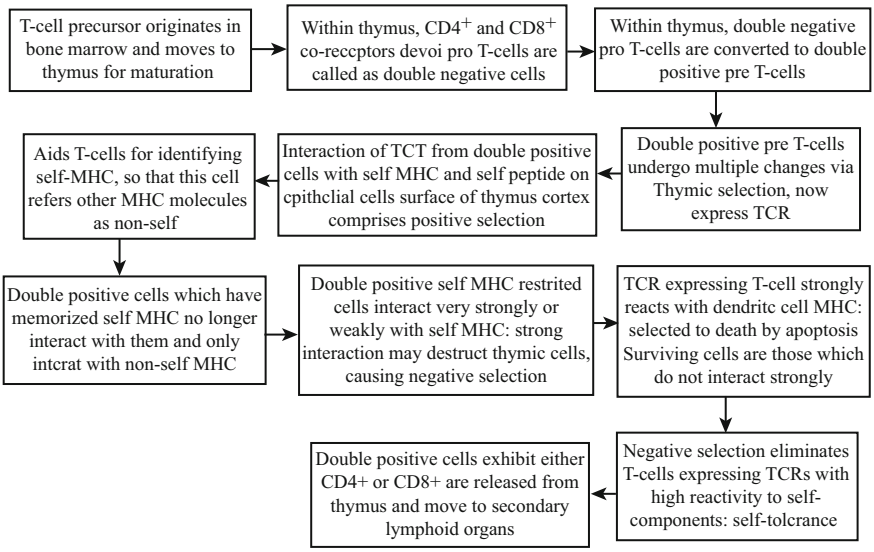

Fig. 19 Chronological steps in T-cell maturation, recognition of self and non-self MHC for their implicit differentiation and tolerance development

9.1 Animals Used in Immunological Research

- Various animals are used in immunological research. The most widely used of these are mice, rats, rabbits, hamsters, sheep, and guinea pigs.
- Blood (which contains immunological cells) is collected from the rabbit's heart or eyes.
- Sheep blood is collected for obtaining red blood cells.
- Mice are broadly used for the isolation of various immunological organs and cells as it is easy to handle and all the reagents and antibodies against mouse cell markers are widely available.
- The most commonly used mice strains are respectively ***BALB/b, BALB/c*** (a pure genetic strain of mice), ***CBA/J, C57BL/6, B6CH-2 Bm12 (Bm12***), etc.
- ***Nude mice, transgenic mice, and knockout mice*** are also used, depending on the experiments.

9.2 Euthanasia of the Animals

- **Euthanasia** (Greek, meaning **"Good Death"**) is termed as the act of humanly conferring painless death to an animal. These procedures are quick, easy to perform, and do not cause physiological or histochemical changes in the cells that may affect scientific results.
- There are specific and detailed guidelines from American Veterinary Medical Association (**AVMA**).

- AVMA Guidelines for the Euthanasia of Animals: 2013 Edition https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.
- These guidelines explain in detail, the procedure of euthanasia in various animals. The reagents and equipment used in these commonly administered euthanasia processes are safe and inexpensive.

  Common techniques used for euthanasia are as follows:
  - Carbon dioxide asphyxiation.
  - Sodium pentobarbital or sodium thiopental overdose.
  - Exsanguinations (to drain of blood).
  - Cervical dislocation.
- While **carbon dioxide asphyxiation** is a painless technique, it may cause toxic effects on the animal tissue(s). **Neonates and fetuses** are resistant to carbon dioxide euthanasia. One should follow the **NIH guidelines for the euthanasia** of rodent neonates and fetuses.
- **Cervical Dislocation and Exsanguinations** have little or no effect on the animal tissues. Cervical dislocation of conscious mice needs technical proficiency to avoid blood rupture.
- All **rodents** (**mice, rats, hamsters, guinea pigs**) must be anesthetized to death if cervical dislocation cannot be performed.
- The acceptable dose for induction of euthanasia in rodents involves the intravenous or intraperitoneal injection of sodium pentobarbital at 50 or greater mg/kg body weight. Intraperitoneal injection of 100 mg/kg ketamine with 10 mg/kg xylazine hydrochloride is also used for mice euthanasia.
- For **non-human primates,** sodium pentobarbital at $\geq$100 mg/kg body weight, is injected intravenously or intraperitoneally for inducing euthanasia.
- Confirmation of complete euthanasia should be verified. Check the following signs of life.
- The animal is not dead unless its heart has stopped beating. Check heartbeat by feeling the vibration on the chest between thumb and forefinger. The eye blink should stop touching the eyeball.
- If the animal is not dead, the process should be repeated.

  NB: Ethical guidelines should be strictly followed while subjecting administering euthanasia to experimental animals. Commercial euthanasia Solution has sodium pentobarbital 390 mg + sodium phenytoin 50 mg/ml.

9.3 Organ Harvesting and Isolation of Immunological Organs

Organ harvesting denotes the surgical removal of organs in a synthetic medium. Normally followed by euthanasia, organs are isolated and harvested from experimental animals in the biosafety hood. Human tissues are collected from any organ after surgical removal of the organ from a patient in a similar fashion as by surgeons in an Operation Theatre. The procedures for humans and animals both are highly aseptic and require a high degree of skill and protective measures for the concerned investigator.

Following ethical guidelines should be strictly followed while handling and collecting organs from mammalian tissues.

- All the surgical instruments must be sterilized before surgery.
- A clean aseptic hood is needed for surgical procedures.
- The desired tissue must be collected aseptically.
- The collected tissues must be gently washed with sterile phosphate buffer saline to prevent contamination.

As discussed before immunological organs are divided into primary and secondary lymphoid organs. Examples of primary lymphoid organs are red bone marrow and thymus respectively, whereas secondary lymphoid organs include the spleen, lymph nodes, Mucosa-Associated Lymphoid Organ**s (**MALT), and Gut-Associated Lymphoid Organs (GALT).

The isolation process for some of the primary and secondary lymphoid organs is described below (Reeves and Reeves 1992).

9.3.1 Isolation of Spleen from Mouse

- **S**pleen is a slightly long, oval; curved, and dark red organ oriented toward the stomach and is placed in the left superior abdomen quadrant.
- Under normal conditions, the spleen is soft, smooth, and convex. Its surface is covered by a thin and transparent capsule while the dorsal side is slightly concave and is connected with the stomach by a gastrosplenic ligament.
- Spleen is a hemato-immunological organ **(a secondary lymphoid organ**), performing significant lymph-erythropoietic functions.
- **Red** and **white pulps are the two parts of the** spleen. Red pulp of the spleen has tissues of erythropoietic function constituted by vessels and cords of various types of red blood cells (hemocytoblasts, erythrocytes), and white pulp has lymphoid tissue with **a high number of stored B and T cells.**
- Spleen is called a secondary lymphoid organ where B lymphocytes, T lymphocytes, and memory cells are stored and utilized. These cells perform important functions as and when infections with the same or different pathogens invade an organism.
- Put the mouse **facing left** so that the spleen can be cut from the backside. Dissect the mouse by opening the skin with a blunt end of scissors and forceps. With forceps, pull the skin backward and pin it up.
- Cut open the membrane with fine end scissors and gently pull outward to make a hole. The red color curved spleen can be easily noticed.
- Pull the spleen from the peritoneum with fine end forceps tearing the connective tissue located behind.
- It is recommended to remove the entire spleen in one go.
- For the large spleen, the top and bottom portions can be held by forceps and torn from the connective tissue, one at a time.
- Took one Petri plate, put the collected spleen into it, and add 5–10 ml Hanks Balanced Salt Solution (**HBSS**) for washing.

- The **spleen of a young, adult 8-week-old mouse weighs approximately 100 mg and measures approximately 15 mm in length, 3 mm in width, and 2 mm in thickness.**

  NB: Under normal conditions, the spleen from a well-rested 6-week-old mouse yields 5–15 × 10^7 live lymphocytes.

9.3.2 Isolation of Thymus from Mouse

- The thymus is a primary lymphoid organ and is involved in the maturation of T lymphocytes.

  The most important functions of the thymus include recognition, identification, and differentiation between self and non-self molecules of the various lymphocytes. Subsequently, it also aids in the tolerance of self-cells containing self-molecules/proteins and the elimination of non-self cells/molecules.
- The thymus in the newborn young, 4–6-week-old mouse has well developed while in an adult 4–6-month-old mice, it becomes **small due to atrophy**, thereby hard to detect. The thymus is yellowish-white and has two lobes, lying close to the base of the heart (Sjodin et al. 1963).
- Normal resting mice have a bigger thymus. A female mouse, in general, has a larger thymus than its male counterpart.
- Make an incision in the chest with the blunt end of surgical scissors beginning at the xiphoid and extending to the neck.
- Crack the ribs with blunt-end scissors and retract with curved forceps.
- The thymus is found **on the median line just under the ribs close to the anterior superior mediastinum, in front of the heart, and behind the sternum.**
- The thymus is a yellowish-white two-lobed organ.
- Pull the thymus away gently by grasping the lobes with curved forceps. The thymus is placed in 5–10 ml HBSS or an appropriate tissue culture medium in a tissue culture plate (Van Alten 1984).
- **Normally from a 6-week-old mouse, 10–30 × 10^7 lymphocytes could be recovered from the thymus.**

  NB: As the thymus is very delicate and soft, extreme care should be taken not to tear it during removal.

9.3.3 Isolation of Lymph Nodes from Mouse

- Lymph nodes are isolated when the mouse is dissected from the front.
- In a normal mouse, the lymph nodes are very small in size and difficult to isolate.
- Take a curved forceps, grasp the lymph node, and pull it free of attached fats and tissues.
- The major lymph nodes prevail in the **auxiliary, cervical, inguinal, and mesenteric regions of the human body**.
- **Axillary nodes** are readily detected; adjacent to brachial nodes, and are located easily in the triceps area.
- Below the large salivary gland and flanking the trachea, **cervical nodes** are located.
- **Inguinal nodes** inside the groin region are buried in a fat pad.

- **Mesenteric lymph** nodes are found in the intestinal mesentery region.
- The lymph nodes are placed in 5–10 ml HBSS or tissue culture medium in a tissue culture plate.
- **Typically, from a normal 6-week-old mouse, the collected lymph node yields 0.5–1 × 10^8 live lymphocytes.**

9.3.4 Isolation of Cells from the Harvested Organs: RBC Lysis from Cell Suspension Using Ammonium Chloride Potassium (ACK) Lysis Buffer

To get RBC-free lymphocytes, the RBC in mouse and human spleen cell suspension are lysed with **Ammonium Chloride Potassium (ACK)** lysis buffer (Table 9).

Procedure

- Treat spleen cell suspension with an equal volume of ACK lysis buffer in a 15 ml tube.
- Keep on ice or at 4 °C for 10 min.
- Lysis of RBC giving a red color solution.
- Make up the volume up to 15 ml with 1× PBS and centrifuge at 1500 rpm for 10 min.
- Wash three times with 1× PBS and discard the supernatant.
- The white pellet is re-suspended with 1× PBS to obtain the RBC-free WBC suspension.

Procedure of RBC Lysis by Ammonium Chloride Method

ACK buffer is not used for lysing RBC in whole blood rather, ammonium chloride lysis buffer is used to lyse RBC from whole blood (Table 10).

NB: *For the working solution, dilute 1 ml 10× concentrate with 9 ml Millipore water. Refrigerate until use.*

- Take 5 ml fresh blood in a heparinized 15 ml tube.
- Put 10 ml cold Ammonium chloride lysis solution and tilt it gently up and down.

Table 9 Preparation of ammonium chloride potassium lysis buffer (1 l)

Chemical name	Amount for 1 l
155 mM NH_4Cl	8.29 g
10 mM $KHCO_3$	1.0 g
EDTA (0.1 mM)	0.2 ml (500 mM)
Water	1 l (final volume)

Table 10 Preparation of ammonium chloride lysis buffer (1 l, 10×)

Chemical name	Amount (1 l, 10×)
NH_4Cl	8.02 g
EDTA	0.37 g
$NaHCO_3$	0.84 g
Millipore water	1 l (final volume)

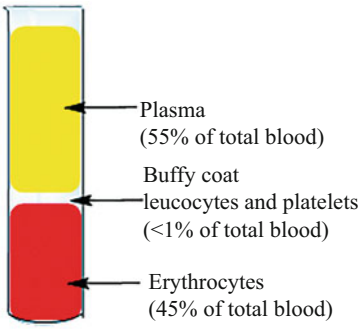

Fig. 20 Human blood fractionated by density gradient centrifugation. Plasma (upper layer), buffy coat (middle, white-colored layer), and erythrocyte or RBC (red blood cell) layer (bottom) can be seen

- Keep for 10 min at room temperature until the liquid is clear red.
- Centrifuge at 4 °C for 10 min at 1500 rpm.
- Decant the supernatant, preserving the white pellet.
- Wash the white pellet three times with 1× PBS.
- Re-suspend the washed pellet finally in 5 ml 1× PBS.
- Count cells by adjusting the cell concentration within **2–4 × 10^6/ml.**

9.3.5 Isolation of Buffy Coat from Blood

- The **Buffy coat** is the thin, white layer fraction between clear plasma and RBC after density gradient centrifugation of the anticoagulant supplemented whole blood sample.
- The Buffy coat consists of less than 1% of the total blood sample volume, consisting of WBC and platelets.
- The Buffy coat is usually white; however, it is sometimes greenish due to the presence of a large number of neutrophils rich in green myeloperoxidase (Fig. 20).
- The Buffy coat of fresh blood is usually worked upon to extract DNA from the blood cells as mammalian RBC is devoid of the nucleus and does not contain any DNA.
- Collect whole blood in a heparinized tube (the tube is pre-treated with anticoagulant heparin).
- Dilute fresh blood with an equal volume of Ca^{+2} and Mg^{+2} free PBS with 2% FBS.
- Centrifuge the tube at 200× *g* for 10 min at room temperature.
- Carefully remove Buffy coat by taking the white layer at the plasma/erythrocyte interface using a Pasteur pipette.

Diagnostic Uses of the Buffy Coat

Enrichment of WBC

In case of extremely low WBC count in blood, a Buffy coat is prepared. A blood smear of this Buffy coat is enriched in the WBC number compared to that obtained from whole blood.

Quantitative Buffy Coat

It is a highly sensitive laboratory test and is preferred for the detection of parasitic infection in blood than the conventional smear test (e.g., malarial parasites). The blood is taken in a tube coated with acridine orange (a fluorescent dye) and a Buffy coat is prepared in that tube. As a result, the fluorescent malarial parasites are observed under UV light at the interface between the Buffy coat and RBC.

9.3.6 Isolation of Cells Using Density Gradient Centrifugation

- Live cells are lighter and dead cells are heavier.
- Similarly "**Larger Cells** are **lighter** and **Smaller Cells** are **Heavier.**"
- These cell properties comprise the principles of density gradient centrifugation.
- Therefore, dead cells fall in the pellet and live cells float at the density and osmotic strength of the solution.

Ficoll-Hypaque Solution

- **Ficoll-Hypaque** is a **water-soluble polysaccharide** and its aqueous solution is used for performing cell separations based on their density.
- Since the density of Ficoll is dependent on temperature, the centrifugation is, therefore, done at room temperature.
- Otherwise, there is a chance of losing lighter cells at the interface, thereafter obtained at the bottom as a pellet.
- **Ficoll is highly toxic to the cells**, as a result of which it is recommended **to wash out the Ficoll from the cells as soon as possible**.

Percoll Gradient

- Percoll is **silica particles coated with polyvinyl pyrrolidone**.
- Percoll solution is preferred over Ficoll as it is **less toxic and has low osmolarity.**
- Both Ficoll-Hypaque and Percoll solutions are used to remove dead cell clumps from an overgrown culture as well as RBC and polymorphonuclear (**PMN**) cell removal from blood.

9.3.7 Isolation of Platelets, Peripheral Blood Mononuclear Cells, Polymorphonuclear Cells, and RBC from Human Blood

- Peripheral blood has various cells namely RBC, WBC, and platelets.
- WBC are further divided into agranulocytes, i.e., monocytes and lymphocytes and granulocytes, i.e., neutrophils, eosinophils, and basophils.
- **Peripheral Blood Mononuclear Cells (PBMCs)** are cells having a single round nucleus. For example, lymphocytes and monocytes.
- **Polymorphonuclear (PMN) cells** are granular and have multiple nuclear lobes. For example, the nucleus of neutrophils has three to seven lobes, eosinophils have two while basophils have one single lobe/nucleus.
- PBMC, PMN, RBC, and platelets are isolated from the human blood by density gradient centrifugation using Ficoll-Hypaque and Percoll solution.

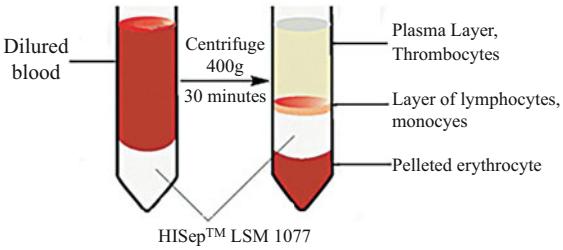

Fig. 21 Isolation of PBMC, PMN, RBC, and platelet sub-components of blood

Procedure for Isolation of PBMC, PMN, RBC, and Platelets

- Dilute the heparinized blood sample using 1:1 with sterile 1× PBS.
- Take 5 ml Ficoll-Hypaque/Percoll solution in a 15 ml sterile centrifuge tube.
- Overlay the diluted blood gently and carefully, over Ficoll solution.
- Centrifuge at 1500 rpm for 30 min at room temperature.
- After centrifugation, the cells are separated into layers based on density. On the top is plasma, with the lightest platelet cells on the uppermost layer followed by a Buffy coat containing PBMCs at the interface and heavier RBC, and PMN cells as a pellet at the bottom (Fig. 21).
- Discard the plasma and collect the Buffy coat containing PBMC in another tube containing 10 ml 1× PBS to remove the Ficoll.
- Centrifuge at 1000–1500 rpm for 10 min and collect PBMC as a cell pellet.
- Wash the PBMC two to three times with 1× PBS and resuspend in complete medium.
- Similarly, wash the pellet containing RBC two to three times with 1× PBS.
- Collect the PMN as a pellet by lysing the RBC and washing further with 1× PBS.
- Re-suspend the PBMC in a 10 ml complete medium before taking an aliquot for cell counting.
- Count the viable cells using **Trypan Blue exclusion method.**
- **Normal yield of PBMC is 0.5–3 × 10^6 cells/ml.**

Enrichment of Polymorphonuclear Cells

- Pellet contains RBC and PMN after centrifugation of blood by Ficoll or Percoll gradient.
- Resuspend the pellet in 5 ml, 1× PBS before treatment with ammonium chloride lysis solution.
- Tilt the test tube and keep it at room temperature for 10 min until the liquid is clear and red.
- Centrifuge at 4 °C for 10 min at 1500 rpm.
- Discard the red supernatant before washing the white pellet thrice with 1× PBS.
- Collect the final pellet as RBC-free enriched PMN cells.

9.3.8 Isolation of Monocytes Using Percoll Gradient

- Prepare Percoll gradient by mixing isotonic Percoll with 10× PBS containing 0.15 M NaCl.

- Three gradients are formed, namely at 60%, 45%, and 25% Percoll extents, respectively.
- Overlay 10 ml 60% Percoll in the lower layer, 20 ml 45% Percoll in the middle, and 10 ml 25% Percoll in the top layer.
- Centrifuge the tube at 6000 rpm in a swing bucket along with 30 min of centrifugation at 4 °C.
- Monocytes can be obtained as 45% Percoll layer, as a white band.
- Discard the top layer.
- Take the monocytes in the middle layer before washing thrice with 15 ml 1× PBS at 1500 rpm for 10 min.
- Monocytes are obtained as **white cell pellets**.

9.3.9 Isolation of Macrophages from Peritoneal Cavity, Spleen, Lymph Node, and Bone Marrow

Isolation of Resident Macrophages from Peritoneal Cavity

The peritoneal cavity denotes the fluid-filled abdominal cavity containing the intestine, stomach, and liver. The most important immunological cells present in the peritoneal cavity are lymphocytes (B/T lymphocytes) and macrophages called **peritoneal macrophages.**

- The peritoneal cavity is considered the primary source of **naïve tissue-resident macrophages** due to the abundance of naïve macrophages. The isolation of peritoneal cavity resident immune cells is challenging due to its fragile structure.
- The following paragraphs describe the isolation, purification, and biochemical characterization of peritoneal resident macrophages.

Materials Required for Isolation of Peritoneal Exudate Macrophage

The following materials are required for isolating peritoneal exudates (resident) macrophages:

- 70% ethanol (disinfecting agent for cleaning various materials including the dissection area, as well as the exterior body parts of the mouse).
- Ice.
- 5 ml syringe with 27 gauge needle.
- 5 ml syringe with 25 gauge needle.
- Sterilized forceps and scissors.
- Dissection tray.
- Styrofoam blocks and pins (use for mounting the mouse).
- Collection tubes (sterile).
- Prechilled sterile PBS with 3% fetal calf serum (**FCS**). Put on ice to remain cool.
- Complete medium with 10% serum and antibiotic, and filter sterilization provision.

Isolation Procedure of Peritoneal Resident Macrophages

This is a standard procedure, well recognized and presently used in many laboratories.

- Sacrifice a euthanized mouse followed by disinfection by spraying 70% ethanol and mounting its back on a **Styrofoam block** in a dissection tray.
- With the help of forceps and scissors, cut the outer skin of the mouse.
- Expose the inner skin lining the peritoneum.
- Do not damage or tear down the inner skin.
- Take a 5 ml syringe with a 27 gauge needle and use it to inject 5 ml ice-cold PBS containing 3% FCS.
- Now gently massage the peritoneum to dislodge any attached cells into the PBS solution.
- To collect the peritoneal fluid, insert a 25 g needle attached to a 5 ml syringe in the peritoneum.
- Collect the peritoneal fluid in the tube kept on ice. In case the needle is clogged by any fat tissues or organs move the needle, a little bit to dislodge the clogging.

  NB: ***The tube is discarded if the cells are contaminated with RBC.***
- To isolate cells from the peritoneal fluid centrifuge it at 1500 rpm for 10 min.
- Discard the supernatant and re-suspend the cells in desired medium or PBS for counting either by hemocytometer or automated cell counter.
- Using this procedure one can isolate approximately 5–10 $\times$ 10^6 million live cells.

  The individual cell percentage may be as follows:
  - 50–60% are B cells
  - 30% are macrophages
  - 5–10% are T cells
- Cells are suspended in a complete medium and are cultured in a culture flask, Petri plates, or 96-well plates.
- The peritoneal cavity B-cell subsets are unique and known as B1 cells in addition to conventional B2 cells. B1 cells are further subdivided into B1a and B1b cells having CD11b and CD5 as surface markers. **B1 cells are autoreactive,** but how they prevent autoimmunity is not yet completely understood. B1 cells produce natural IgM antibodies, providing early protection against pathogens. $CD5^+$ B1a cells produce IL-10 and have some negative regulatory properties. Peritoneal cavity B1 cells are an interesting cell population to study due to their functional diversity and roles in development and immunity regulation.

9.3.10 Isolation of Thioglycollate or Starch Elicited Activated Macrophages

- Use thioglycollate or starch solution to get the activated peritoneal macrophages.
- This method yields ten times higher activated macrophage recovery than the resident macrophages, from the peritoneal fluid.
- Inject 5 ml, 3% (w/v) Brewer thioglycollate solution or 4% starch solution in the peritoneal cavity of each mouse. Five mice are kept in the animal house after the challenge.

- Sacrifice the mice on the fourth day after which the peritoneal cells are collected, as mentioned above.
- The activated macrophages obtained by this procedure are different from the resident macrophages as they are physiologically more active.

9.3.11 Isolation of Macrophages from Spleen (Adherence Method)

- Take out the spleen in a 5 ml sterile 1× PBS.
- Macerate or homogenize the spleen to get a single-cell suspension.
- Put 5 ml ACK solution and mix it for RBC lysis.
- Incubate for 10 min and make up to 15 ml using 5 ml PBS.
- Centrifuge in a 15 ml centrifuge tube at 1500 rpm for 10 min.
- A white pellet and red transparent supernatant are obtained.
- Decant the red supernatant and wash the white pellet once with 1× PBS for 10 min.
- Re-suspend the white pellet in 10 ml complete medium.
- Count the cells using a hemocytometer and adjust cell count to $\mathbf{2 \times 10^6/ml}$.
- Dilute 1:10 with complete medium and put in tissue culture Petri dish.
- Incubate for 1–2 h or overnight at 37 °C in a CO_2 incubator.
- Wash out mildly with warm PBS thrice to remove non-adherent WBCs.
- Add 10 ml of complete medium right away.
- A monolayer of adherent macrophages is obtained.

9.3.12 Isolation of Macrophages from Lymph Node

- Take out the lymph nodes in 5 ml sterile 1× PBS.
- Macerate or homogenize lymph nodes to get a single cell suspension.
- Add 5 ml ACK solution and mix it for RBC lysis.
- Incubate for 10 min and makeup to 15 ml using 5 ml PBS.
- Centrifuge in a 15 ml centrifuge tube at 1500 rpm for 10 min.
- A white pellet and red transparent supernatant are obtained.
- Repeat steps as mentioned above.

9.3.13 Isolation of Macrophages from Bone Marrow

- Take out the long bones, i.e., femurs in a sterile fashion, inside a Petri dish.
- Cut the side of the bone with scissors.
- Holding one side with forceps, flush the bone with 2 ml medium in a syringe fitted with a 26 g needle and repeat.
- Put 5 ml ACK solution and mix it for RBC lysis.

Enrichment of T Cells, B Cells, Macrophages, Dendritic Cells, and Natural Killer Cells

Enrichment of T Cells and B Cells and Macrophages

- The white pellet harvested from the spleen, lymph node, or bone marrow is a heterogeneous mixture of adherent macrophages and dendritic cells and non-adherent T cells, B cells, NK cells, etc.

- Common cell surface markers **for macrophages, T cells,** and **B cells** are **Mac-1, Thy1.2, and μ chain of IgM**. **Dendritic cells** have a **$CD11C^+$ marker,** used for identification as well as separation of cells from a white blood cell mixture.

Enrichment of Macrophages by Adherence

- White blood cells or WBC are adhered in tissue culture Petri dishes to get macrophage monolayer.
- The supernatant contains non-adherent cells, namely T cells, B cells, and NK cells which are discarded.
- The remaining adherent monolayer consists of 99% macrophages.
- These macrophages are confirmed to have a surface **Mac-1 marker.**

Enrichment of Macrophages by Cell Removal Method

- **5×10^6 spleen cells/ml** are incubated with 3 μg anti-μAb (to eliminate B cells) plus anti Thy1.2 (to eliminate T cells) Ab and complement.
- The mixture is incubated at 37 °C for 30 min.
- The B cells and T cells are lysed to give a macrophage-enriched WBC population.
- The average yield of the enriched macrophage population is 99%.
- These macrophages are **non-specific, esterase positive,** and confirmed to have surface **Mac1 and F4/80 markers, using fl**ow cytometry.

Enrichment of T Cells Using Cell Removal Method

- WBCs are incubated with anti μ (to eliminate B cells) Ab plus anti-Mac-1 (to eliminate macrophages) Ab with complement.
- The mixture is incubated at 37 °C for 30 min.
- The B cells and macrophages are lysed to give a T-cell-enriched population of WBCs.
- The average yield of the enriched T cells population is 98%.
- These T cells are confirmed to have surface **Thy1.2 marker** as confirmed by flow cytometry.
- The T-cell-enriched preparation responded to **ConA** but not to **LPS** in cell culture.

Enrichment of B Cells by Cell Removal Method

- WBCs are incubated with anti Thy1.2 (to eliminate T cells) Ab plus anti-Mac-1 (to eliminate macrophages) Ab plus complement.
- The mixture is incubated at 37 °C for 30 min.
- The T cells and macrophages are lysed to give a B cell-enriched population of WBCs.
- The average yield of enriched B cells population is 97%.
- These B cells are confirmed to have surface **μ marker** as confirmed by Flow Cytometry.
- B cell-enriched preparation responded to **LPS** but not to **ConA** in cell culture.

Magnetically Driven Isolation

Chapter ▶ "Isolation and Purification of Various Mammalian Cells: Single Cell Isolation" presents a comprehensive discussion on a magnetically activated cell sorter (**MACS,** For **MACS** methodology, follow the manufacturer's protocol).

10 Culture of Immunological Cells

10.1 Cell Culture Medium

- The complete medium consists of either **RPMI 1640** or Dulbecco's Modified Eagle's Medium (**DMEM**) supplemented with 10% heat-inactivated fetal calf serum (FCS) or fetal bovine serum (FBS), 12 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM Na-pyruvate, and 50 μM 2-ME (**mercaptoethanol)** for quick growth). pH should be adjusted between 7.2 and 7.4.
- **Make a complete medium, filter sterilize, aliquot, store at 4 °C, and use within a week.**

  NB: For T-cell culture, the addition of 2-ME is mandatory while for other cell cultures, it is optional and can be skipped.

10.2 Murine Macrophage Culture

1. Sacrifice the mice and harvest organs or peritoneal macrophages.
2. Isolate peritoneal macrophage or macrophage from spleen/lymph node/bone marrow.
3. Otherwise, start with PBMC isolated from blood.
4. Resuspend the cells in a 10 ml complete medium.
5. Count cells using a hemocytometer and adjust cell count to 2×10^6/ml.
6. Dilute 1:10 with medium and put in tissue culture Petri dish.
7. Incubate for 1–2 h or overnight at 37 °C, 5% CO_2 in a CO_2 incubator.
8. Wash out mildly using warm PBS thrice to remove the non-adherent WBCs.
9. Add 10 ml of complete medium, right away.
10. A monolayer of adherent macrophages is formed and could be confirmed using an inverted microscope.
11. These monolayers of macrophages can be tested as **Mac1 positive in flow Cytometry or confirmed via non-specific esterase staining**.
12. These macrophage monolayers can be activated via **500 ng/ml LPS** or other cytokine or drugs of interest.
13. After treatment, wash the macrophage monolayer with warm PBS thrice and add a complete medium.
14. Every alternate day, take out 5 ml spent medium and feed with 5 ml complete medium.

The macrophages can be cultured for 1–5 days at 5% CO_2 as required in the treatment protocol (Zhang et al. 2008).

10.3 Human Monocyte-Derived Macrophage Culture

The monocytes are differentiated into macrophages, which are further de-differentiated with altered morphology, gene expression, and functions. Two different types of environments aid this macrophage differentiation, which are as ahead.

10.3.1 M-CSF + Fetal Bovine Serum (FBS) Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) + FBS

Here is a brief discussion about them.

Using M-CSF + GM-CSF

Selection of Human Monocytes

The protocol is obtained from the work of* Lacey et al. (2012) *with minor modifications.

This type of macrophage is also called as M1 type.

Human monocytes are purified from the Buffy coats using the Rosette Sep Ab mixture, which negatively selects $CD14^+$ monocytes. In the next step, the monocytes would be purified via Ficoll-Hypaque density gradient centrifugation.

Culture of the Cells

The monocytes would be cultured in RPMI 1640, supplemented with 10% heat-inactivated FCS, 2 mM GlutaMax-1, 100 U/ml penicillin, and 100 μg/ml streptomycin, stimulated with M-CSF (2500 U/ml), human GM-CSF (5 ng/ml), or both, for appropriate periods, or cultured for 7 days in M-CSF to differentiate them into MDM GM-CSF and further into GM-MDM, or in both CSFs to differentiate them into (M-CSF + GM-CSF)-MDM (Lacey et al. 2012).

Using M-CSF and IL-10

The protocol is obtained from the work of* Jin and Kruth (2016) *with minor modifications.

This type of macrophage is also called as M1 type.

Experimentally, it is noticed that the use of human serum rather than FBS generates the GM-CSF type regardless of whether M-CSF or GM-CSF is included in the differentiation medium.

Careful morphological observations revealed that M-CSF-type macrophages tend to be more elongated than GM-CSF type, which resembles **fried eggs** in their morphology.

Isolate **PBMCs** from human blood using Ficoll-Hypaque density gradient centrifugation.

1. Complete medium composition is RPMI 1640 medium with 2 mM L-glutamine, 50 ng/ml M-CSF, 25 ng/ml interleukin-10 (IL-10) and 10% FBS.
2. Plate PBMC in complete medium for 2 h in T25 culture flasks kept at 37 °C and furnished with 5% CO_2.
3. Wash non-adherent cells with a warm medium and add a complete medium.
4. Monocyte monolayer is formed.
5. Scrape monocytes and incubate in a 48-well plate at a concentration of **1×10^5 cells/well** in RPMI supplemented with **autologous human plasma**.
6. The monocytes are cultured for 6–7 days at 37 °C and in a 5% CO_2 environment to obtain macrophage-like morphology.
7. Confirm the monocyte-derived macrophages as $CD14^+$ (CD14 is a molecular marker of monocytes) using Flow Cytometry (Jin and Kruth 2016).

Using Autologous Human Serum

1. The Ficoll-Hypaque density gradient centrifugation is used to isolate PBMCs from human blood.
2. To culture these cells, the following medium composition is used: RPMI 1640 medium with 2 mM L-glutamine and 10% FBS.
3. Culture PBMC in complete medium in T25 culture flasks at 37 °C, in a 5% CO_2 environment for 2 h.
4. Wash non-adherent cells with a warm medium before adding a complete medium.
5. Monocyte monolayer is formed.
6. Scrape monocytes and incubate in a 48-well plate at a concentration of **1×10^5 cells/well** in RPMI supplemented with **autologous human plasma**.
7. The monocytes are cultured for 6–7 days at 37 °C and in a 5% CO_2 environment to obtain macrophage-like morphology.
8. Confirm the monocyte-derived macrophages as $CD14^+$ (CD14 is a molecular marker of monocytes) using Flow Cytometry (Gupta et al. 2001).

10.4 Infection of Splenic Macrophage Culture by Leishmania Parasite

1. Sacrifice BALB/C mice, isolate spleen and harvest splenic mononuclear cells.
2. Make an RBC-free spleen cell suspension at **1×10^6 cells/ml**.
3. Make a complete cell culture medium with the following composition: RPMI 1640 medium, 10% heat-inactivated FCS, 12 mM HEPES, and 50 U gentamicin/ml.
4. Take a 60-mm-diameter Petri dish and adhere 4 ml spleen cell suspension at 37 °C, in a 5% CO_2 environment.
5. Culture for 2–3 h.
6. Following culture, wash the Petri dish with warm RPMI 1640 medium to remove non-adherent cells.
7. Adherent cells are >99% splenic macrophages.
8. Incubate the splenic macrophages in a complete medium.

9. For in vitro infection, add **5 × 10^7 *Leishmania donovani* parasites** to the macrophage culture at a 20:1 parasite/macrophage ratio.
10. Make macrophage cultures without parasites in parallel to control normal macrophages.
11. Incubate Petri dishes with macrophages plus parasites at 37 °C for 4 h to allow for parasite internalization.
12. Following 4 h of incubation, wash the cells thoroughly using warm RPMI solution to remove free parasites.
13. Now incubate it with the complete medium at 37 °C for different durations (12, 18, and 24 h).
14. Observe infected macrophage cultures under an inverted microscope (Mukhopadhyay et al. 2000).
15. Collect the culture supernatant at 12, 18, and 24 h for nitrite and tumor necrosis factor-alpha (TNF-α) assay (Murray et al. 1982).
16. Wash the infected macrophages at 12, 18, and 24 h with 1× PBS.
17. Air-dry the infected macrophages, fixed with methanol and stained with Giemsa stain.
18. Count the % infected cells and amastigotes/100 macrophages under an oil immersion microscope.
19. Calculate the phagocytic index as % of infected macrophages × number of amastigotes/macrophages.

10.5 Mouse Dendritic Cell Culture

1. Inject mouse spleen with 0.5 ml collagenase (400 U/ml). Otherwise, filter the cell debris from spleen cell suspension using a cell strainer.
2. Adhere **1 × 10^7 cells/ml** in 100 × 15 cm tissue culture Petri dishes at 37 °C and 5% CO_2 environment for 2 h.
3. Remove the non-adherent cells and wash adherent cells 2× with a warm medium by gently swirling and pooling the non-adherent cells in a test tube.
4. Place **2 × 10^6** non-adherent cells in 10 ml complete medium in tissue culture-treated culture flask, add 20 ng/ml GM-CSF, and incubate overnight at 37 °C and in a 5% CO_2 environment.
5. Take out 50% medium on every alternate day, add 50% fresh complete medium with 20 ng/ml GM-CSF and incubate overnight at 37 °C in a 5% CO_2 environment.
6. Take out and add medium very gently so that the loosely adherent dendritic cells are not disturbed.
7. Change medium on days 3, 5, and 7 as mentioned in step 5. On day 8 or 9, harvest the dendritic cells as mature cells.
8. Culture cells as mentioned in step 4 in a non-tissue culture-treated flask or Petri dish to obtain immature dendritic cells on day 8 (Gorak et al. 1998).

NB: As dendritic cells mature by adhering to a plate in presence of GM-CSF, non-tissue culture treated 96-well u-bottom plate is used to get immature dendritic cells, thereby preventing an early maturation.

10.5.1 Isolation of Dendritic Cells by Flow Cytometry

On the overnight dislodging of transiently adherent cells, enrich for dendritic cells through negative selection. These cells are a mixture of dendritic cells and F4/80 positive macrophages.

- Treat these **5×10^6** transiently adherent cells with an **anti-μAb** (to eliminate B cells), **3 μg/ml goat anti-mouse anti-Thy 1.2 antibodies** (to eliminate T cells), and **3 μg/ml goat anti-mouse F4/80** antibodies (to eliminate loosely attached macrophages). Finally, incubate for 1 h at room temperature or 37 °C, in a 5% CO_2 environment.
- On completion of 1 h, centrifuge the cells at 1000 rpm for 10 min and resuspended in 1 ml non-toxic baby rabbit complement (diluted 1:2).
- Now incubate for 1 h at room temperature or 37 °C and in a 5% CO_2 environment.
- Centrifuge the cells and remove the supernatant.
- Wash the cells and stain with **anti-CD11C$^+$ FITC conjugate**. Partially enriched dendritic cells contain 10–50% **CD**11C$^+$ **cells** as confirmed on being stained with anti-CD11C$^+$ FITC conjugate.
- Purify cells further via cell sorting using FACS Caliber. Sorted cells contain >90% CD11C$^+$ dendritic cells.
- For some experiments, dendritic cells are sorted into **CD8α$^+$ and CD8α$^-$** sub-populations by treating with labeled antibodies to CD11C, MHC class II, and CD8α.

10.6 Human Dendritic Cells Culture

1. The Ficoll-Hypaque density gradient centrifugation is used to harvest PBMCs from human blood.
2. Take a T25 flask and plate the PBMC in a complete medium at 37 °C, in a 5% CO_2 environment for 2 h.
3. Wash non-adherent cells with warm and complete media.
4. After 48 h, wash unbound and loosely adherent mature dendritic cells and collect them for further use.
5. Monocyte monolayer is formed.
6. In the next step, the monocytes would be treated with dendritic cell growth factors, **20 ng/ml recombinant human GM-CSF** and **20 ng/ml recombinant human IL-4**, for 6 days in a humidified incubator at 37 °C, in a 5% CO_2 environment.
7. Change half the medium every 2 days with a complete fresh medium carrying the step 6 growth factors.

8. Check the monocyte differentiated dendritic cells on 6 days of culture via staining with anti-HLA-DR Ab and anti-CD11C Ab, followed by Flow Cytometry confirmation. It is likely that 60–70% of cells are monocyte differentiated dendritic cells and are immature.
9. Treat monocyte differentiated immature dendritic cells with a complete medium supplemented with growth factors mentioned above plus LPS (500 ng/ml) and culture for 48 h for further maturation.
10. After 48 h, mature human dendritic cells are generated ex vivo from human monocytes (Sachdeva et al. 2015).

10.6.1 Primary T-Cell Culture and T-Cell Proliferation (Radioactive Method)

1. Treat the spleen/lymph node/bone marrow cells to deplete RBC using ACK buffer. Adhere cells in 100 × 15 cm tissue culture Petri dish or flask for 2 h and take the non-adherent cells in another tube.
2. Discard the adherent cells.
3. Enrich the T cells via removal as mentioned above. T cells should be Thy 1.2 positive, as confirmed by Flow Cytometry.
4. T cells should be cultured in a 96-well flat bottomed cell culture plate at **2×10^5 cells/well** for 72–96 h, carrying a complete medium supplemented with 2-ME and L-glutamine.
5. Activate the T cells with **3 μg ConA** (concanavalin A) or **25 μg/ml KLH** (keyhole limpet agglutinin) for 72–96 h.
6. Observe T-cell blast in the culture after 48 h.
7. For the T-cell proliferation assay in the last 24 h, add **1 μCi/well ^{3}H thymidine (6.7 Ci/mmole)** to the culture.
8. T-cell proliferation is measured using **^{3}H thymidine uptake**.
9. Harvest the proliferating T cells using a cell harvester. Now, cut and transfer the membrane to the glass vials, add 5 ml scintillation fluid to the vial and measure the radioactive count in a β-counter (Roy et al. 1987, 1989).

10.6.2 T-Cell Culture and Proliferation (Non-Radioactive Method)

The protocol is obtained from the research works of Manna and Frazier (2003) with minor modifications.

1. Take 96-well cell culture plate. Treat some of the wells with either soluble or bound antibodies against CD3 or CD47.
2. Plate **5×10^3** murine/human T cells in 100 μl complete medium, in a 96-well flat-bottom plate
3. Incubate for 96 h at 37 °C, in a 5% CO_2 environment.
4. Observe T-cell blasts in the culture after 48 h, under the microscope.
5. After 72 h, measure cell number using the Cell Titer 96 aqueous non-radioactive cell proliferation kit (Promega, Madison, WI).
6. The result of this essay depends on the intact functional mitochondria (Manna and Frazier 2003).

10.7 Type 1 and Type 2 T Helper Cell Culture

The protocol is obtained from the research work of Radiah and colleagues (2003) with minor modifications.

1. Take DO-11.10 BALB/C transgenic mice specific for a TCR, recognizing OVA323–339.
2. Sacrifice the mice.
3. Isolate the spleen and lymph nodes.
4. Use the spleen and lymph nodes to isolate naive $CD4^+$ T cells through negative selection using anti-$CD8^+$ and anti-MHC II microbeads (Miltenyi Biotec, Auburn, CA) as per the manufacturer's instructions.
5. Sacrifice non-transgenic BALB/c mice, isolate the spleen, and irradiate the spleen cell suspension in a culture flask at 3000 rad (cesium source).
6. Plate the naive 5×10^6 $CD4^+$ T cells from transgenic mice and APC from non-transgenic mice in a 1:1 ratio plus OVA 323–339 (1 μg/ml, Research Genetics) in a complete medium containing 10% heat-inactivated FBS.
7. Add IL-12 (10 ng/ml), anti-IL-4 (1 μg/ml), and rIL-2 (10 U/ml) on days 1 and 3 for the development of Th1-polarized population.
8. Add rIL-4 (10 ng/ml), anti-IL-12 (2 μg/ml), anti-IFN-γ (1 μg/ml), and human rIL-2 (10 U/ml) on days 1 and 3 for T_h2-polarized cells.
9. Culture the cells at 37 °C, in a 5% CO_2 environment.
10. On day 6, centrifuge cells on a Ficoll step gradient. Wash and harvest the cells for re-stimulation. Repeat the treatment as mentioned in step 5.
11. Separately re-stimulate a portion using irradiated BALB/c APCs and 1 μg/ml OVA323–339 peptide to determine the polarization of each population.
12. Harvest the culture supernatants at 72 h. Measure IL-4 and IFN-γ in the supernatant using ELISA.
13. The T_h1 and T_h2 supernatants typically contain more than 1000 ng IFN-γ/ml and less than 0.04 ng IL-4/ml and less than 3 ng IFN-γ/ml and more than 4 ng IL-4/ml respectively.
14. Harvest IFN-γ and IL-4 producing cells as T_h1 and T_h2 cells.
15. The cultured cells are strongly polarized to the T_h1 or T_h1 phenotype.
16. Freeze the T_h1 and T_h2 cells for future use. After thawing re-stimulate cells in culture as mentioned in step 5 (Radiah et al. 2003).

10.8 Cytotoxic T-Cell Culture

Cytotoxic T cells are $CD8^+$, generated in mice immunized with either allogeneic tumor cells as antigen or any other protein antigen. The cytotoxic cells are isolated and cultured in vitro using a mixed leukocyte reaction, optimum for use via cell-mediated cytotoxic assay.

10.8.1 Mixed Leukocyte Reaction

- A mixed leukocyte reaction (**MLR**) could be performed with cytotoxic T cells.
- For culture, use DMEM with L-asparagine (36 rag/l), L-glutamine (216 rag/l), L-arginine HC1 (116 rag/l), 10 μM 2-ME and 5% FBS.
- For the source of **responding** and **stimulating cells,** spleens of the appropriate strains are used.
- **Responding cells:** Harvest spleen cells from normal mice or mice immunized 2–4 months earlier with a single intraperitoneal injection of 30×10^6 living allogeneic tumor cells as antigen. The immune spleen cell populations exhibit very low cytotoxic activity remaining after the in vivo immunization.
- **Stimulating cells:** Isolate spleen cells from syngeneic mice and irradiate the cells with 1000 Rads dose of (Cesium source), immediately before culture.
- **Responding cells:** In a 30 ml, tissue culture flask, add 20 ml MLR medium. To this, mix $\mathbf{25 \times 10^6}$ responding cells with an equal number of irradiated stimulating cells.
- Incubate the culture flasks upright at 37 °C, in a 5% CO_2 environment for 4 h.
- Collect and wash cells 1× with medium, re-suspend to 5×10^6 viable cells/ml using Trypan blue exclusion test.
- The below-described cytotoxic assay system can be used (*Cerotinni JC* et al*, 1974*).

10.8.2 Cell-Mediated Cytotoxicity Assay

The research protocol described here was initially established by Karanikas et al. (1999***).***

1. Mix ^{51}Cr-labeled target (T) cells 1×10^4 with effector (E) cells at different **E:T ratios** in a 200 μl final volume, having a complete medium in round-bottom 96-well microtiter plates.
2. Incubate the tubes with 5% CO_2 for 4 h at 37°C.
3. After 4 h, harvest 100 μl supernatant aliquots in another 96-well plates.
4. Take radioactive count in a gamma counter.
5. **MAXIMUM RELEASE:** Maximum ^{51}Cr release was obtained by incubating target cells with 1% Triton-x-100.
6. **SPONTANEOUS RELEASE:** Spontaneous ^{51}Cr release was measured with medium alone, being normally <10% maximal release in all experiments.
7. The percent-specific lysis can be calculated as follows:
   $\%^{51}$Cr-release = (Experimental release − Spontaneous release)/(Maximum release − Spontaneous release) × 100 (Karanikas et al. 1999**)**.

10.8.3 Immunization of Mice with Antigen

- Immunize 6–8-week-old female BALB/C (H-2^d) and C3H/HeJ (H-2^k) mice with 0.2 ml intraperitoneal injection of 50 μg KLH emulsified with complete adjuvant.
- Perform two booster immunizations every 2–3 weeks.
- Perform assay after 14 days.
- Control mice are injected with phosphate-buffered saline (PBS) in adjuvant.

10.8.4 Preparation of Lymphoid Cell Suspensions

- Prepare single-cell suspensions from spleen and mesenteric, axillary, and cervical lymph nodes after mice sacrifice.
- Lyse RBC in single-cell suspension with 0.83% NH_4Cl for 4 min at 37°C.
- Re-suspend 8×10^6 lymphocytes in complete medium supplemented with 10% FCS.
- Cell viability of >98% using Trypan blue dye exclusion, is attained.
- Harvest $CD8^+$ T cells by MACS method using positive selection with anti-$CD8^+$ Ab magnetic beads *(Dynal or Miltenyi Biotech)*.
- These harvested cells are stimulator cells.

10.8.5 Mixed Leukocyte Reaction

- Harvest $\mathbf{4 \times 10^6}$ spleen cell suspension from naïve mice.
- Irradiate the spleen cells with 3000 rad (cesium source).
- Plate $\mathbf{8 \times 10^6}$ effector cells/well (stimulator cells) in complete media in a 24-well plate and culture with $\mathbf{4 \times 10^6}$ syngeneic irradiated cells (responding cells).
- Add 0·5 mg/ml KLH to the stimulator cell suspension ($\mathbf{20 \times 10^6}$ **cells/ml**).
- Incubate cultures for 7 days at 37 °C and in a 5% CO_2 environment.
- Take out half of the spent medium and add fresh complete medium every alternate day.

10.8.6 Target Cells

- Label $\mathbf{1–3 \times 10^6}$ cells with 100–200 μCi radioactive sodium chromate in a 0.5 ml Tris phosphate-buffered saline, supplemented with 5% heat FBS.
- Target cells were labeled with ^{51}Cr via incubating with a 10 ml complete medium.
- Wash the cells 3× after 30–45 min of incubation at 37 °C.
- Mix labeled cells with target cells. Tumor cell lines syngeneic to DBA/2 (P-815-X2 mastocytoma, L-1210 lymphoma), BALB/C (MOPC-315 plasmacytoma), and C57BL/6 strains (EL4 leukemia, GIL4 lymphoma) are maintained in the ascitic form in mice of relevant strain as well as in the in vitro environment.

10.8.7 ^{51}Chromium Release Assay by Cytotoxic T Cells

- Cytolytic activity of cytotoxic $CD8^+$ T cells is measured via a ^{51}Cr release assay.
- Incubate ^{51}Cr-labeled, 1×10^4 target cells (T) with effector cells (E) at different E to T stoichiometries, in a 200 μl final volume of culture medium, in round-bottomed 96-well microtiter plates for 4 h at 37 °C and 5% CO_2 environment.
- After 4 h at 37 °C, harvest 100 μl supernatant aliquots in another round-bottomed 96-well plate.
- Ascertain the radioactive count in a gamma counter.
- The results from triplicate wells are expressed as the mean percentage of specific chromium release.
- Maximum ^{51}Cr release was obtained by incubating target cells with 1% v/v Triton-x-100 (maximum release).

- Spontaneous ^{51}Cr release is measured with medium alone (spontaneous release). In general, the spontaneous release is <10% maximal in all experiments.
- **The percent-specific lysis was also calculated as %^{51}Cr-release = (Experimental release − Spontaneous release)/(Maximum release − Spontaneous release) × 100.**

10.8.8 T Regulatory Cells or Suppressor T-Cell Culture

- The T regulatory **(T_{reg})** cells are previously known as ***suppressor T cells.***
- The basic function of T_{reg} is to maintain **self-tolerance** and thereby **prevent autoimmunity**.
- These cells are CD4 positive.
- Thus, the regulatory cells are derived from the ***same lineage as those of naïve CD4$^+$ cells***.
- These cells also produce CD25$^+$ as surface biomarkers while FOXP3$^+$ is an intracellular marker.
- Several inhibitory cytokine productions are a characteristic feature of these cells.
- Such cytokines include transforming growth factor-beta (TGF-β), IL-35, and IL-10.
- Generally, these cells are immunosuppressive and down-regulate the induction and proliferation of effector T cells.
- Regulatory T cells can sometimes stimulate other cells to express IL-10 (Bettelli et al. 2006; Curiel 2007).

10.8.9 Expansion and Culture of Regulatory T Cells In Vitro

The culture of T_{reg} mentioned here was initially described by Tang et al. (2004), and later on, Siemasko et al. (2008) also described a protocol.

Here is a brief discussion of the protocol:

1. Sacrifice C57BL/6 or BALB/c mice, before isolating superficial cervical lymph node and spleen.
2. Harvest spleen cells and lymph nodes by macerating gently between the ends of two sterile frosted slides.
3. Mouse T_{reg} cell isolation kit is utilized to isolate CD4$^+$CD25$^+$ T cells (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol.
4. Expand regulatory T cells in vitro according to modified protocol, as demonstrated by *Tang and colleagues.*
5. Prepare a complete RPMI-1640 medium by adding the following: 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 50 μM 2-ME, 1 mM sodium pyruvate, 0.01 mM non-essential amino acids and 10 mM HEPES.
6. Put the medium in 24-well plates and culture the isolated CD4$^+$CD25$^+$ regulatory T cells at 2×10^6 cells/2 ml/well.
7. Henceforth, append anti-mouse CD3-Ab with anti-mouse CD28 Ab coated 4 μm polystyrene magnetic beads in 1:1 stoichiometry (Dynal, Invitrogen,

Carlsbad). Thereafter, include mouse rIL-2 to each well (20 ng/ml; R&D Systems, Minneapolis, MN).

8. Preserve the culture at 0.7–1 × 10^6 cells/ml via diluting with an IL-2 complemented complete medium for 8–12 days.
9. Remove all magnetic beads in culture using a magnet at the end of the seventh day.
10. Ensure the T_{reg} cells in culture using the implicit $CD4^+$, $CD25^+$ marker, and intracellular $Foxp3^+$ prevalence through Flow Cytometry.
11. Visualize the supernatants from in vitro T_{reg} cell culture, using a bioassay (Luminex Corp., Austin, TX) for requisite extents of TGF-β and IL-10 cytokine (Siemasko et al. 2008; Tang et al. 2004).

Flow Cytometry of Regulatory T Cells

- This technique is popularly known as fluorescently activated cell sorting (**FACS**).

  This research protocol was originated from the work of Siemasko et al. (2008) with modifications.

- ***The FACS analysis of T_{reg} cells could be accomplished by using the following steps:***

  1. Obtain 5 × 10^5 cells in a 100 μl FACS buffer (PBS, 0.02% sodium azide, and 2% bovine serum albumin, BSA).
  2. To establish the surface prevalence of $CD4^+$ and $CD25^+$ receptors. Initially, incubate 5 × 10^5 cells with 1 μg/tube of purified anti-mouse CD16/32 (BD-Pharmingen, San Diego, CA) Ab at 4 °C (maintained on ice) for 10 min. Such a treatment gets rid of the possible F_c binding of the primary antibodies.
  3. Nurture 5 × 10^5 T cells along with 1 μg biotin rat anti-mouse $CD4^+$ (clone GK 1.5) Ab and PE-labeled anti-mouse Cd25 (IL-2 receptor α chain, p55, clone PC61) Ab for half an hour at 4 °C. It is recommended to use 1 μg isotype control antibodies biotin rat IgG2b, κ and PE rat IgG 1, λ respectively.
  4. Subject the T-cells to washing, twice with FACS buffer followed by their re-suspension at 5 × 10^5 cells/100 μl buffer.
  5. Stain the tubes carrying biotin-labeled antibodies with 1.5 μl accessory staining pigment (Streptavidin PerCP, BD-Pharmingen) followed by ice-incubation for 20 min in the dark.
  6. To conduct intracellular Foxp3 staining, the T cells are re-suspended in 350 μl, 2% methanol devoid formaldehyde. This is succeeded by nurturing till 15 min at 4 °C in the dark.
  7. Wash the cells again in FACS buffer followed by their re-suspension in 0.5% saponins (Sigma-Aldrich grade).
  8. Add 1 μg anti CD16/32 Ab per tube of T cells and ice-incubate for 10 min in the dark. Supplement 1 μg/tube of FITC anti-mouse Foxp3 (clone PCH101, eBiosciences, San Diego, CA) or 1 μg/tube of FITC rat IgG2a isotype (eBiosciences) control to the apt cell groups before being nurtured yet again on ice medium till 30 min in the dark environment.

9. Wash the T cells with 1 ml, 0.5% saponins buffer, and re-suspend in the 500 μl Flow Cytometry buffer. Probe the $CD4^+CD25^+$ surface and intracellular $FoxP3^+$ expression of T_{reg} cells using Flow Cytometry (FACS Calibur; BD Biosciences, Mountain View, CA) using Cell Quest software (Siemasko et al. 2008).

Suppression Assays by Regulatory T Cells In Vitro

1. Add graded numbers (1:1. 1:2. 1:4, 1:8, 1:16, 1:32, 1:10, and 1:100) of expanded or freshly sorted regulatory T-suppressor cells to 5×10^4 $CD4^+$ T cells (responder cells) in a U-bottomed 96-well plate.
2. Irradiate separately, the spleen APC in a culture flask at 2000 rad.
3. Stimulate the culture with 5×10^4 irradiated splenic APC and 1 μg/ml anti-CD3 antibody.
4. Stimulate $CD4^+$ T-cell cultures without regulatory T cells in the same manner as positive controls.
5. Stimulate the co-culture with anti-CD3 as described above or with 0.1 μg/ml OVA peptide, when $CD4^+$ T cells from DO11.10 TCR transgenic mice are used as responder cells.
6. Maintain the co-culture at 37 °C in a 5% CO_2 environment for 72 h.
7. In the last 18 h, add 1 μCi/well ^{3}H-thymidine in the culture so that proliferating cells are radio-labeled.
8. Harvest proliferating T cells using a cell harvester, cut and transfer the membrane to the glass vials, add 5 ml scintillation fluid in the vial and measure the radioactive count in a β-counter.
9. As an alternative non-radioactive method, label the responding $CD4^+$ cells with 2.5 μM CFSE before the suppression assay. Measure the proliferation level after 72-h co-culture through Flow Cytometry ascertained CFSE dilution.

10.9 NKT Cell Culture

The information provided here is from the research works of Kinjo et al. (2006) and Bendelac et al. (2007).

- Nearly unchanged natural killer T (NKT) cells are the typical T lymphocytes having the characteristic features of innate and adaptive immune cells. These perform the duties analogous to a bridge facilitating interactions between adaptive and innate immunity.
- These cells are CD1d-restricted T cells responding to a range of glycolipids. Such cells bear an intensely specific T-cell receptor (TCR) gamut and mature in the thymus on getting activated from a common precursor of CD4/CD8 double-positive thymocytes.
- NKT cells generate IFN-γ, IL-4 and IL-17 cytokines.

- Varying as per the specified TCR pattern, the NKT cells can be traditional (more commonly referred to as type I or invariant NKT cells (iNKT cells) and non-traditional (type II NKT cells).
- The α-galactosylceramide (α-GalCer) is frequently employed as a model antigen to screen the iNKT cell duties, wherein non-classical MHC class I molecule CD1d harbors α-GalCer and associated glycolipid antigens to iNKT cells (Kinjo et al. 2006; Bendelac et al. 2007).

10.9.1 Human iNKT Cells Isolation

Human Vα24*i* NKT cell lines are generated from blood, using the following procedures:

- Obtain blood samples from healthy male and female subjects.
- Isolate PBMCs from the blood.
- Re-suspend 2 × 10^6/ml PBMC in complete medium in a culture flask.
- Activate PBMC with 100 ng/ml α-GalCer and incubate for 24 h at 37 °C and in a 5% CO_2 environment.
- On completion of 24 h, add human 30 ng/ml rIL-2 (Biolegend) to the culture and incubate at 37 °C, in a 5% CO_2 environment.
- After 10–15 days, sort cultured NKT cells using the 6b11 (Becton Dickinson) antibody.
- For loading, pulse PBMCs 100 ng/ml of α-GalCer (KRN7000, Avanti Polar Lipids), for 4–5 h and incubate at 37 °C.
- Irradiate this PBMC loaded with α-GalCer at 3000 rads and wash with the medium.
- Re-stimulate the sorted cells with washed and irradiated PBMCs loaded with αGalCer.
- Add washed irradiated PBMC to sorted NKT cells in a 5:1 ratio and incubated at 37 °C in a 5% CO_2 environment for 24 h.
- After 24 h, add 30 ng/ml human rIL-2 to the culture, in presence of α-GalCer.
- Expand the cells for the next 10–14 days.
- After 10–14 days, harvest the cell population as human Vα24i NKT cells (Pulido et al. 2018).

10.9.2 Mouse iNKT Cells Isolation

- Harvest liver of three C57BL/6J mice.
- Resuspend the cells in a 50 ml RPMI-1640 medium.
- Centrifuge at 60 g for 5 min at 4 °C and separate the 45 ml supernatant.
- Centrifuge at 600 g, 4 °C for 8 min, and isolate the non-parenchymal cells in supernatant.
- Take the pellet, re-suspend in medium, and overlay in 10 ml, 37.5% Percoll.
- Centrifuge at 850 g, 4 °C, for 30 min in **"break off"** mode.
- Resuspend the pellet in 2 ml RBC lysis buffer (sterile) and incubate for 5 min at RT.
- Wash cells with PBS two times and add 1 ml of complete RPMI 1640 media.

- Centrifuge cells at 480 g for 8 min at 4 °C.
- Resuspend the pellet in complete RPMI 1640 culture media.
- Harvest viable cells by staining with LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit L-3495, Life Technologies) to exclude dead cells. Alternatively, staining with propidium iodide (PI) to exclude dead cells.
- Stain the viable liver cell suspension with CD3-FITC^{+}, mCD1d-PBS-57-PE^{+}.
- Sort the iNKT cells using Flow Cytometry in a FACSAria III (Becton Dickinson) Obtain primary mouse CD3^{+}, CD1d + iNKT cells (Kim et al. 2017a; Pulido et al. 2018).

10.9.3 Co-Culture Preparation of Splenic Dendritic and Vα14I NKT Cells and Dendritic Cells

1. Sacrifice BALB/c or C57BL/6 mice and harvest spleen.
2. Treat the spleen either with 100 U/ml collagenase or macerate with sterile frosted glass slides to get a uniform spleen cell suspension.
3. Prepare RBC-free spleen cell suspension.
4. Make a Percoll gradient of (60%, 50%, and 40%), overlay the spleen cell suspension, and centrifuge at 1670 g for 20 min.
5. Collect cells as the low-density fraction (40% and 50%), and wash 2× to remove Percoll.
6. Plate the cells in a tissue culture flask or Petri dishes in a complete medium and incubate for 2 h at 37 °C and in a 5% CO_2 environment.
7. After 2 h, remove non-adherent cells and culture adherent cells at 37 °C, in a 5% CO_2 environment for 24 h.
8. After 24 h, collect loosely adherent cells like dendritic cells and select positively using MicroBead-conjugated CD11c mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) and Super MACS (Miltenyi Biotec).
9. Pulse the splenic dendritic cells with 100 ng/ml, α-GalCer for 3 h, wash and collect as α-GalCer pulsed dendritic cells (Watarai et al. 2012).

10.9.4 VA14I NKT Cells

- Harvest spleen from mice and make spleen cell suspension.
- Isolate the splenic Vα14i NKT cells through positive selection with PE-conjugated CD1d/α-GalCer tetramers and MicroBead-conjugated anti-PE antibody (Miltenyi Biotec).

10.9.5 Setting Up Co-Culture of A-GALCER Pulsed Dendritic Cells and NKT Cells

- Culture 5×10^4/100 μl splenic α-GalCer pulsed Vα14i NKT cells with 5×10^4 splenic α-GalCer pulsed dendritic cells in a complete medium in a 96-well, round-bottom culture plate in presence of α-GalCer (100 ng/ml).
- Incubate for 24 h at 37 °C, in a 5% CO_2 environment.
- After 24 h, collect the supernatant. Measure IFN-γ and IL-4 levels using ELISA (OptEIA ELISA set; BD Pharmingen).

10.9.6 Isolation and Purification of Mouse NKT Cells

In 2001, the initial studies of Gapin* et al. *help to formulate the following research protocol:

1. Sacrifice male and female C57BL/6 mice for 8–12 weeks and harvest spleen.
2. Prepare single-cell suspension from spleens.
3. Incubate spleen cell suspension with anti-CD19 magnetic beads (Miltenyi Biotec) in MACS buffer for half an hour to get rid of B cells. Subsequently, the passage of the cells through MiniMACS columns (Miltenyi Biotec) and retrieving the flow through.
4. The NKT cells are stained using allophycocyanin (APC) or Pacific Blue-conjugated murine CD1d tetramers followed by configuring the $Cd4^+$ T cells with murine anti-CD4 Ab conjugated magnetic beads. This $Cd4^+$ T-cell sorting is accomplished through a positive selection MACS enrichment kit, following the manufacturer's guidelines (Miltenyi Biotec).

   Sort out NKT and CD4 cells using FACS Aria II (BD Biosciences) (Gapin et al. 2001).

10.9.7 NKT and $CD4^+$ Cells Co-Culture

The typical culture medium employed for co-culturing the NKT and $CD4^+$ cells are the RPMI medium 1640, carrying 10% FBS, 2 mM glutamine, and penicillin/streptomycin

1. Stimulate the sorted NKT and Cd4 cells using the plate-bound anti-CD3 (2.5 μg/ml) and soluble anti-Cd28 (2.5 μg/ml) antibodies (eBioscience) for an optimum time in the existence of IL-2 (10 U/ml) in a complete medium. This is followed by nurturing the cells at 37 °C, in a 5% CO_2 environment.
2. Stimulate NKT cells with α-GalCer (100 ng/ml) to study activated NKT cells not possessing $CD4^+$ T cells, on the surface.
3. Analyze cells from step 1 to step 2 separately using FACS Aria II (BD Biosciences) as mentioned above.

   After interaction with CD1d in the thymus, the immature double-positive thymocytes generate the expanded NKT cells. These NKT cells comprise both, $CD4^+$ and double negative lymphocytes in the thymus and vicinity wherein α-chain is also expressed. Such observations implicitly infer a prevalence of double-positive intermediate for CD1d receptive NKT cells (Gapin et al. 2001).
4. Significant heterogeneity prevails in the invariant natural killer T (iNKT) cells, concerning prevalences of CD4 and IL-17 receptor B (IL-17RB, a receptor for IL-25, a prominent factor regulating T_H2 immune response. Notably, IL-17RB^+ and IL-17RB^- subsets, comprise the two subtypes of iNKT cells. The IL-17RB^+ subtypes are subsequently distinguished into $CD4^+$ and $CD4^-$ subtypes, viz-a-viz thymus and vicinity expressions. The $CD4^+$ IL-17RB^+ *i*NKT cells generate T_H2 (IL-13), T_H9 (IL-9 and IL-10), and T_H17 (IL-17A and IL-22) cytokines in response to IL-25, in a manner dependent on E4BP4. On the contrary, the $CD4^-$ IL-17RB^+ *i*NKT cells comprise a retinoic acid receptor relayed orphan receptor (ROR)γt^+ subset, generating T_H17 cytokines, on being stimulated with IL-23 in a typically, E4BP4 independent manner (Watarai et al. 2012).

10.10 Functional Assay of NKT Cells

In 2013, Lee et al. described the functional assay of NKT cells. Further in 2019, Kumar et al. further modified the research protocol.
Here is the protocol for the functional assay of NKT cells:

1. Plate 1×10^6 cells of total thymocytes or thymocytes depleted CD8- and CD24-positive cells using MACS in complete RPMI 1640 medium.
2. Treat unstimulated or stimulated NKT cells with 50 ng/ml PMA (Sigma Aldrich) and 1.5 μM ionomycin (Sigma Aldrich) in the presence of Monensin (3 μM, Sigma-Aldrich) or using Golgi Plug (BD Biosciences) for 4 h.
3. Permeabilize the cells using Cytofix/Cytoperm Plus (BD) for intracellular cytokine staining.
4. Treat NKT cells with intracellular cytokine staining according to the manufacturer's instruction (BD Biosciences) for detection of intracellular cytokine expression.
5. Confirm NKT cells as CD1d tetramer-positive and intracellular cytokine IFN-γ, IL-4, and IL-17 positive cells through FACS involving Flow Cytometry.
6. Cell viability is determined via ascertaining propidium iodide uptake in Flow Cytometry (Lee et al. 2013; Kumar et al. 2019).

10.11 Flow Cytometry Assays

The protocol described here is obtained from the research work of Kumar et al. (2019) with minor modifications.

Typical fluorescent antibodies in use for the surface and intracellular staining in the presence of anti-FcγR mAB (2.4G2), are as follows: anti-mouse TCR-β (H57-597) Pacific Blue/APC, PBS-57-loaded Cd1d tetramer APC/PE/Pacific Blue, anti-mouse CD4 (GK1.5) APC-Cy7, anti-mouse IFN-γ (XMG1.2) PE/FITC, anti-mouse IL-4 (BV D6-2462) PE-Cy7 and anti-mouse IL-17 (TC11-18H10) PerCP-Cy5.5 (all from eBioscience).

1. Confirm NKT cells as CD1d tetramer-positive and intracellular cytokine IFN-γ, IL-4, and IL-17 positive cells through Flow Cytometry using FACS.
2. Cell viability is determined through ascertaining propidium iodide uptake via Flow Cytometry.
3. Acquire cells on FACS Canto II (BD Bioscience), performing data analysis using Flow Jo (TreeStar software v9.9) (Kumar et al. 2019).

10.12 Actions of γδ T Cells

- γδ T cells are recognized innate immune cells
- These cells respond more rapidly than the adaptive immune T_h17 cells to protect pathogen-infected hosts.

- Additionally, these cells exhibit pattern recognition receptors (PRRs) on their surface and secrete TLR2 on the surface as one of the PRRs.
- In γδ T cells, IL-23 activates TLR2 which secretes IL-17.
- IL-17 is classically defined by its ability to induce the expression of a variety of pro-inflammatory mediators, ultimately leading to neutrophil recruitment and activation at the inflammation site.

  NB: High-level secretion of IL-17 is associated with several inflammatory disorders, including psoriasis.

10.13 Isolation and Culture of γδ T Cells

In 2015, Beck* et al. *described the isolation and culture of γδ T cells. The original protocol may change a little bit in line to suit the experimental purposes.

- The requirement of expansion and activation cultures for cytotoxicity analysis and immunotherapy purposes are met through the spleen cells harvested from C57BL/6 TCRβ-deficient ($TCR\beta^{-/-}$) mice models ($B6.129P2^{Tcrbtm1Mom/J}$) which are devoid of αβT cells.
- Isolate spleens from C57BL/6 TCRβ-deficient ($TCR\beta^{-/-}$) mice and macerate to form spleen cell suspension.
- Isolate PBMC using Ficoll gradient centrifugation.
- Set spleen cell cultures at 5×10^6 cells/ml density in a complete medium.
- Relocate the spleen cells into the tissue culture wells, priorly coated with rat anti-mouse CD2 mAb clone RM2–5 (BD Biosciences).
- Supplement the mouse rIFN-γ (1000 U/ml) and rIL-12 (10 U/ml) to the culture. Thereafter, nurture for 24 h at 37 °C, in a 5% CO_2 environment.
- Add 3× fresh culture medium to the well after 24 h.
- Expose the cultures with 10 ng/ml anti-CD3 mAb clone 145-2C11 (BD Biosciences) and 300 U/ml murine rIL-2 (R&D Systems).
- Gradually substitute 50% spent medium with equivalent fresh complete one, having 10 U/ml IL-2 (Roche Diagnostics) every 3 days.
- Harvest the γδ cells from the eighth day onward. Thereafter, stain these with the conjugated hamster anti-mouse antibodies CD3-allophycocyanin (clone 145-2C11) and γδ TCR –FITC (clone GL3; all procured from BD Biosciences).
- Examine the purity of γδ T cells via Flow Cytometry using a FACS Calibur flow cytometer (BD Biosciences).
- Stain cells with propidium iodide uptake to determine the viability, using Flow Cytometry.
- All γδ T cells are CD3 positive and γδ TCR positive (Beck et al. 2015).

10.14 Intracellular Staining of Cytokines

- After surface staining, fix the γδ T cells and permeabilize in 250 μM. Cytofix/Cytoperm (BD Biosciences) at 4 °C.
- Wash γδ T cells 2× with BD Perm/Wash buffer.
- Label γδ T cells with antibodies for IL-17-PerCP-Cy5.5, IFN γ-PECy7, and IL-4-APC.
- Analyze cells by Flow Cytometry by a FACS Canto analyzer (BD Biosciences) using FACS Diva software.
- γδ T cells are positive for intracellular cytokines, IL-17, IFN γ, and IL-4.
- γδ T cells have $CD4^+$ $CD8^-$ phenotype and express CD25, CD38, CD71, and HLA-DR as activation antigens (Beck et al. 2015; Raziuddin et al. 1992).

10.15 In Vitro γδ T-Cell Culture Assay

Serum amyloid A1 (SAA1) is a prominent acute-phase protein generated exclusively in the liver. The SAA tempts T_h17 cell differentiation alongside the IL-17 oozing in T_h17 and γδ T cells.

- Use the 7–9 weeks old (age-matching) SAA1 transgenic mice in the C57BL/6 setting, which characteristically over-express the SAA1 gene. Within the lymph nodes, IL-17 generating γδ T cells ($IL\text{-}17^+$ γδ TCR^+ cells), are rich sources of T cells.
- Use age-matched (7–9 weeks old) SAA1 transgenic mice in C57BL/6 background that over-express SAA1 gene.
- Isolate spleens, thymus, and lymph nodes of C57BL/6J mice.
- The γδ T cells could be isolated using magnetically activated cell sorting, following the manufacturer's protocol (MACS, Miltenyi Biotech, San Diego, CA).
- The isolated γδ T cells (2×10^5 cell/100 μl) would be cultured in a complete medium in the presence of 50 ng/ml rIL-23 and 2 μg/ml rSAA1 for 4 h at 37 °C and in a 5% CO_2 environment.
- After 4 h, collect cells for confirmation as γδ T cells by flow cytometry.
- Propagate the culture for up to 48 h at 37 °C, in a 5% CO_2 environment.
- After 48 h determine the cell viability and harvest supernatant for ascertaining IL-17 expression.
- ***Readers are suggested to go through the optimized protocols for γδ T-cell expansion and lentiviral transduction (Choi et al. 2019; Wang et al. 2019).***

10.16 Splenic B Cells Culture

- Sacrifice naïve BALB/c or C57BL/6 mice, isolate spleen and harvest spleen cell suspension.
- Treat spleen cell suspension with ACK buffer to remove RBC.

- Take RBC devoid spleen cell suspension.
- Purify naive mature B cells through negative selection from RBC lysed spleen cell suspensions using MACS anti-mouse CD43 microbeads (no. 130-049-801; Miltenyi Biotec) on a Midi-MACS magnetic separation apparatus (Miltenyi Biotec).
- Culture purified B cells in B-cell medium (sterile-filtered RPMI 1640 [no. 22400–089; Life Technologies] plus 15% FBS [no. 35-010-CV; Corning] plus 1% additional L-glutamine [200 mM stock] plus 1% penicillin/streptomycin mixture [no. 400-109; Gemini] plus 0.0005% 2-ME [no. BP176-100; Fisher Scientific]) at 37 °C and in 5% CO_2 environment.
- Stimulate 0.5×10^6 B cells/ml with various conditions: LPS (20 mg/ml, no. L4130; Sigma-Aldrich), LPS ([20 mg/ml] plus IL-4 [12.5 ng/ml, no. 404-ML-010; R&D Systems]), LPS ([20 mg/ml] plus IL-2 [100 U/ml, recombinant human; National Institute of Health] plus IL-5 [5 ng/ml, no. 215-15; PeproTech]), LTD (LPS [10 mg/ml] plus TGF-β [2 ng/ml, no. 240-B-010; R&D Systems] plus anti-IgD-dextran [0.33 mg/ml, FinaBio no. 0001; Fina Bio-solutions]), anti-CD40 ([0.5 mg/ml, clone HM40–3, no. 16-0402-86; eBioscience] plus IL-4 [12.5 ng/ml]), and anti-CD40 ([0.5 mg/ml] plus IL-4 [12.5 ng/ml] plus IL-5 [2 ng/ml]). Culture cells at 37 °C and in a 5% CO_2 environment.
- Split cells 1:2 at 48–72 h. Flow cytometric analysis of CFSE staining is performed according to the manufacturer's instructions (Cell-Trace CFSE Cell Proliferation Kit Protocol; Invitrogen).
- The frequency of follicular B cell (IgM^{int} IgD^{+} CD^{21+} CD^{23+}) and marginal zone (IgM^{+} IgD^{-} CD^{21hi} CD^{23lo}) B cells, is observed in the normal spleen as confirmed by Flow Cytometry.
- Check induction of characteristic plasma blast markers such as CD^{138} and intracellular Blimp1 (the master transcriptional regulator of the plasma lineage) for indicating the presence of antibody-secreting splenic plasma cells (Pucella et al. 2019; Nutt et al. 2015).
- For intracellular staining of Blimp1, Bcl^6, and Ki^{67}, fix the cells and permeabilize using Bio-Legend True-Nuclear Transcription Factor Buffer Set (no. 424401).
- To stain for intracellular Ig, fix cells, and permeabilize using BD Biosciences CytoFix/CytoPerm Fixation/Permeabilization Solution Kit (no. 554715), omitting the GolgiStop step.

10.17 Mouse Natural Killer Cells Culture

The protocol described here is obtained from the research work of Manna et al. (2010) with minor modifications.

1. Sacrifice 4–6-week-old female C57BL/6 mouse, harvest spleens and prepare spleen cell suspension.
2. Plate 5×10^6 splenocytes on Petri dishes and incubated in a complete medium for 2–3 h at 37 °C and in a 5% CO_2 environment
3. After 2–3 h, discard the adherent cells and pool the non-adherent cells in a test tube.

4. NK cells are isolated by negative selection.
5. For isolation, treat 1×10^7 non-adherent cells with anti-mouse pan T-cell antibody jIj10, anti-mouse B-cell antibody B220, anti-mouse macrophage antibody F4/80 plus goat anti-mouse polyvalent Ig coated magnetic beads (Dynal, Oslo, Norway).
6. Repeat 3×, the treatment of antibodies with beads.
7. Wash with PBS to remove antibodies and beads. Keep the cells.
8. Centrifuge the cells, more than 98% of which are viable (Trypan blue exclusion test).
9. Confirm cells, **PK136 (NK1.1)** marker positive NK cells as checked by immunofluorescence via FACScan (Becton Dickinson, CA) using cell quest software (Manna et al. 2010).

10.18 Natural Killer Cell Culture

The protocol described here is obtained from the research work of Manna et al. (2010) with minor modifications.

1. Isolate PBMCs from the blood of a healthy donor using Ficoll-Hypaque density gradient centrifugation.
2. Plate PBMC in complete medium in a Petri dish before incubating in a complete medium for 2–3 h.
3. Remove non-adherent cells, washing 3× with PBS.
4. Use non-adherent cells for NK cell isolation.
5. Treat 1×10^7 non-adherent cell suspension with purified monoclonal antibodies to CD3, CD14, CD19, CD20, CD83, and Dynabeads.
6. Repeat the antibody and bead treatment 3×.
7. Wash cells 2× and harvest cells as 94% viable cells (Trypan blue exclusion test).
8. Confirm NK cells as CD56 positive, using anti-human CD56 antibody by FACScan (Becton Dickinson, CA) using cell quest software (Manna et al. 2010).

10.18.1 Culture of Allogeneic Natural Killer Effector Cells with the Infected Mouse Macrophages

The protocol described here is obtained from the research work of Manna et al. (2010) with minor modifications.

- Culture macrophages from BALB/c mice on glass coverslips in a complete medium.
- Infect the macrophages with Leishmania parasites in 1:10 proportion.
- Prepare NK cells from allogeneic mice.
- Culture infected BALB/c macrophages (target cells) on glass cover-slips with allogeneic NK cells (effector cells) in a total volume of 1 ml **at 50:1 effector to target (E:T) stoichiometry for 48 h** in complete medium at 37 °C and in 5% CO_2 environment.
- After 48 h, wash the coverslips with PBS, and fix and stain with Giemsa.

- Observe infected macrophages under an oil immersion microscope.
- Count at least 600 macrophages with a quadruplicate set in each treatment.
- Determine Anti-leishmanial activity in terms of the % infected macrophages, and the number of intracellular Leishmania parasites per 100 macrophages.
- Phagocytic index of macrophage = % infected macrophages × number of intracellular Leishmania parasites/macrophages (Manna et al. 2010).

10.18.2 Transwell Experiment of *Leishmania Donovani* Infected Macrophages with Natural Killer Cells

The protocol described here is obtained from the research work of Manna et al. (2010) with minor modifications.

1. Culture macrophages from BALB/c mice on glass cover-slips in a complete medium.
2. Infect the macrophages with Leishmania parasites in 1:10 stoichiometry.
3. Place the coverslips in complete medium, having 24-well plates with Transwell chambers.
4. Perform Transwell experiment using 24-well plates with costar Transwell chambers (0.4 μm pore size, corning).
5. Place NK cells in Transwell chambers in 600 μl total volume, placed above the cover-slip, and incubate for 48 h at 37 °C, in a 5% CO_2 environment.
6. Wash the coverslips, and fix them with acetone, followed by staining with Giemsa.
7. Observe the infected macrophages under an oil immersion microscope.
8. Determine the antileishmanial activity of NK cells as described above (Manna et al. 2010).

10.19 Isolation of Neutrophils and Eosinophils

The protocol described here is obtained from the research work of Kim* et al. *(2017b) with minor modifications.

Neutrophils and eosinophils are the prominent granulocytes commonly derived from myeloid progenitors through successive stages, in the bone marrow. Neutrophil primary granule proteins are called NPGPs and eosinophil-specific granule proteins are called ESGPs. ESGPs are major basic protein 1 (MBP1), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) respectively. NPGPs azurophilic proteins are of three kinds, namely proteinase 3, myeloperoxidase, cathepsin G (*CTSG*), and neutrophil elastase, respectively.

1. Collect the human peripheral blood.
2. Isolate PBMC using Ficoll-Hypaque or Percoll gradient (1.070 g/ml) centrifugation method.
3. Wash PBMC 3× with PBS to remove Ficoll/Percoll.
4. Select cells by negative selection using anti-CD16 monoclonal antibody-conjugated microbeads (Miltenyi Biotech).

5. Anti-CD16 antibody treatment removes NK cells, neutrophils, monocytes, and macrophages.
6. Check the purity of neutrophils and eosinophils to >95%, as evidenced by **Diff-Quick Giemsa staining** (Kim et al. 2017b).

10.19.1 Differentiation of Human PBMC to Neutrophil and Eosinophil Culture

The protocol described here is obtained from the research work of Kim* et al. (2017b) *with minor modifications.

1. Collect human peripheral blood.
2. Isolate PBMC by Ficoll-Hypaque or Percoll gradient (1.070 g/ml) centrifugation method.
3. Wash PBMC 3× with PBS to remove Ficoll/Percoll.
4. Now, MACS $CD34^+$ MicroBead kit is used to purify $CD34^+$ cells from mononuclear cells (Miltenyi Biotech, Auburn, CA, USA).
5. In the next step, culture $CD34^+$ cells for 6 days in IMDM medium containing the following: 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, cytokine cocktail of stem cell factor (SCF), Flt-3 ligand (Flt-3L), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-5.
6. On the sixth day, plate cells in 12-well plates in IL-3 and IL-5 supplemented medium and culture for another 6 days.
7. On the 12th day, change 50% medium and add fresh complete medium every alternate day for further 6 consecutive days.
8. On the 12th day, NPGPs reached a peak expression and thus, sharply declined. **Cultured cells resemble neutrophils at this stage.**
9. On the 18th day, culture the cells in IL-5 supplemented medium for additional 12 days, with 50% medium change every 3 days.
10. ESGPs exhibit the expression from the 18th day onward, in the cultured cells.
11. Cells are differentiated toward eosinophils, within 24-day culture period.
12. On the 24th day, **cultured cells resemble eosinophils**.
13. Wash eosinophils with PBS followed by air-drying and fixation with methanol. Thereafter, stained with Giemsa.
14. Observe eosinophils granule formation and bilobed nucleus shape under an oil immersion microscope.
15. All ESGPs, i.e., MBP1, EPX, ECP, and EDN, are most abundantly expressed on the 24th day of culture, in differentiated eosinophils as compared to day 18 (Kim et al. 2017b).

10.20 Mast Cells Isolation from Mouse Spleen

1. Isolate spleen from infected BALB/c mice and prepare spleen cell suspension.
2. Purify mast cells from the spleen cell suspension by depletion of macrophages, B cells, and T cells, as per protocol demonstrated by ***Wells and Mann***.

3. Overlay the resulting spleen cell suspension on a Percoll-gradient and centrifuged at 2000 rpm for 15 min at 4 °C.
4. Recover and wash the cells in the pellet. Now re-suspend in RPMI-1640 complete medium.
5. Harvest the mast cell count and re-suspend in 1×10^7 cells/ml, in a complete medium.
6. Stain mast cells with Giemsa-colophonium or alcian blue-Safranin stain before counting the mast cells.
7. Immunostain the mast cells using the antibodies against mast cell protease-I and protease-II.
8. Irrespective of the staining method, mast cell property remains the same (Ashman et al. 1991; Wells and Mann 1983).

10.21 Mast Cells Culture, Degranulation, and Generation as Supernatant

1. Culture 0·5 ml of 1×10^7 cells/ml mast cell suspension in a culture flask in complete medium with 0·5 ml serum drawn from 120 days *Leishmania donovani* parasite infection of BALB/c mice, having 25 μg/ml crude soluble leishmanial antigen at 37 °C and in 5% CO_2 environment.
2. After 48–72 h, collect the activated mast cell supernatant (Saha et al. 2004).

10.22 Determination of Mast Cells Supernatant Anti-Leishmanial Activity on Infected Macrophages Culture

Culture 1×10^4 macrophages on glass coverslips in 35 mm sterile Petri dishes with mast cell supernatants for 24 h at 37 °C and a 5% CO_2 environment.

1. After 24 h, initiate the infection using 1:10 macrophage: Leishmania parasites.
2. Add 1×10^5 Leishmania parasites to 1×10^4 macrophages. Incubate for 6 h at 37 °C and in a 5% CO_2 environment.
3. Wash 3× with a warm medium to remove excess parasites before retrieving the infected macrophages. Add complete medium.
4. Add activated mast cell supernatant to the infected macrophages.
5. Co-culture the infected macrophages with activated mast cell supernatant for 72 h at 37 °C and in a 5% CO_2 environment.
6. After 72 h, cells are washed with PBS, fixed with methanol, and stained with Giemsa.
7. Count the infected macrophages under an oil immersion microscope (Saha et al. 2004).

10.22.1 Mouse Platelet Isolation

1. Anesthetize mouse/mice using ketamine.
2. Collect blood from the tail vein or heart into syringes containing 1 ml acid/citrate/dextrose (12.5 g/l sodium citrate, 10.0 g/l D-glucose, and 6.85 g/l citric acid).
3. Add to 6 ml PIPES buffer (150 mM NaCl and 20 mM PIPES, pH 6.5).
4. Centrifuge at 100 g for 15 min.
5. Collect the platelet-rich supernatant.
6. Add 1 U/ml apyrase enzyme and 1 M prostaglandin E1 (final concentrations) to the supernatant.
7. Centrifuge at 1000 g for 10 min.
8. The platelet pellet is re-suspended in the medium before counting the platelets under a microscope (Crist et al. 2013).

10.22.2 Human Platelet Activation

1. Blood is withdrawn from volunteers in the EDTA test tube.
2. Centrifuge at 100 g for 10 min to obtain platelet-rich plasma (refer to Sect. 9.3.5).
3. Transfer the platelet-rich plasma to another tube and centrifuge at 1300 g for 4 min.
4. Re-suspend the platelets in Tyrode's solution-HEPES buffer, at the rate of 2×10^8/ml (Table 11).
5. Seed 1×10^8/ml platelets per well in complete medium.
6. Activate platelets with ADP at the rate of 50 μmol/l.
7. For negative control, culture platelets with an anti-activation cocktail (Table 12).
8. Culture for 6 h in a 5% CO_2 environment.
9. Harvest the supernatant from activated platelets and use supernatant for further study (Cha et al. 2000).

Table 11 Tyrode's solution-HEPES buffer

Materials	Concentrations (mmol/l)
NaCl	150
KCl	2.5
$MgCl_2$	01
$CaCl_2$	02
D-glucose	5.5
HEPES	2.5
$NaHCO_3$	12

NB: Adjust pH to 7.4 before adding 1 mg/ml BSA

Table 12 Anti-activation cocktail

Materials	Concentrations (mmol/l)
Aspirin	01
Theophylline	01
Prostaglandin E_2	10 nml/l

10.23 Differentiation of Human CD34$^+$ Megakaryocyte Progenitor Cells In Vitro

The protocol is obtained from the research works of Crist et al. (2008) with modifications.

1. Blood is withdrawn from healthy donors.
2. Isolate CD34$^+$ cells by MACS from G-CSF immobilized blood (Miltenyi Biotech).
3. To induce differentiation along the megakaryocyte lineage, culture the CD34$^+$ cells for 14 days in SCF and TPO (Stem Cell Technologies, Vancouver, Canada).
4. Separation of the CD41$^+$ and CD41$^-$ populations is accomplished using CD41$^+$ magnetic bead separation (Miltenyi Biotech, Auburn, CA).
5. Determine the percentage of CD41/61$^+$ cells through Flow Cytometry (Crist et al. 2008).

10.24 Differentiation of Mouse LSK Hematopoietic Progenitor Cells

The protocol is obtained from the research work of Crist et al. (2008) with modifications.

1. Sacrifice five to seven C57BL/6 mice and harvest femur bones.
2. With the help of magnetic bead positive and negative selection, isolate mouse Lin$^-$, Sca-1$^+$, c-Kit$^+$ (LSK) hematopoietic stem cells from C57BL/6 bone marrow (Miltenyi Biotech).
3. For 12 days, culture the pure LSK cells in a Stem span medium carrying recombinant TPO, interleukin-1 (IL-1), IL-3, and SCF (PeproTech, Rocky Hill, NJ).
4. Separate primary murine megakaryocytes from mouse bone marrow. The culture-derived megakaryocytes are distinguished from non-megakaryocyte cells using anti-CD41 microbeads (Miltenyi Biotech) (Crist et al. 2008).

11 Usefulness of the Work on Immunological Cells and Organs

Analysis of immune cells is required for the following purposes.

Flow Cytometric Analysis
Single-cell analysis.
Monoclonal Antibody Production
Western Blot Analysis

Distinctive from the cell line scenario (such as EL4, etc.).
Cell Proliferation Assays
Radioactive/non-radioactive.
Colony Formation
In Vitro Differentiation Assays
$CD4^+$ helper cell differentiation.
Cytotoxicity Assay
Mixed Leukocyte Reaction
Cytokine Production
Cell-Based Assay
This makes use of phagocytosis, respiratory burst, intracellular signaling, adhesion and migration by chemotaxis, etc.
In Vitro Infection by Microbes.

12 Conclusions

Immunological cells protect our body from the non-self alien particles and disease-causing pathogens such as viruses, bacteria, fungi, and protozoa. The process of conferring resistance and further protection from the disease-causing pathogens is called **"immunity."** While **innate immunity** is inherited from mother to child, every newborn mammal gradually develops a specific type of immunity, as and when a specific pathogen enters the body, termed **acquired immunity. All the innate and acquired immunological cells are developed from red bone marrow ($CD34^+$ cells).** Immunological cells can be therefore isolated from primary lymphoid organs such as red bone marrow, thymus, and secondary lymphoid organs such as the spleen, lymph nodes, MALT, and GALT. This chapter described the isolation of various lymphoid organs such as the thymus, spleen, and lymph nodes as well as various immunological cells from MALT, GALT, and blood. Isolation, purification, enrichment, and culture of the innate immunological cells described include **monocytes/macrophages** recovery from various regions, such as **dendritic cells, natural killer (NK) cells, and several others**. Additionally, isolation, purification, enrichment, and culture of acquired immunological cells, i.e., both **B cells** and various **T cells (T helper, T cytotoxic)**, are described. Additionally, certain mixed cell cultures are also described here. The chapter completes with a very brief description of the isolation and culture significance of different mammalian immunological cells.

13 Cross-References

► Culture of Neuron and Glia Cells
► Isolation and Primary Culture of Various Mammalian Cells
► Stem Cell Culture and Its Applications

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Culture of Neuron and Glia Cells

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https://doi.org/10.1007/978-981-19-1731-8_10-2

Abstract

The mammalian nervous system consists of neurons that transmit the coordinated neuronal signals throughout the body and glia or supporting cells that support, protect, and nourish the neurons. Adult neurons lose the capacity to divide due to the absence of centrioles. The supporting or glia cells which are around ten times in number than neuronal cells continuously divide throughout the lifetime. However, new neurons arise from neural stem cells (NSCs) during embryogenesis and are preserved even in adults within the specified brain regions such as the subventricular zone and other sections of the nervous system. Additionally, neuronal cell lines are derived from the neuronal tumor/cancer cells that have a capacity for continuous divisions. Immortal cell lines are characteristically different than NSCs. The present chapter describes the basics of isolation and primary culture of neuronal cells, particularly NSCs from various brain regions such as the hippocampus, striatum, and cerebellum. The culturing methods of primary neuronal stem cells and various neurological cell lines such as PC-12 and HEK-293 are discussed. Additionally, isolation and culture of glia or supporting cells, particularly microglial cell culture, have been included.

Keywords

Neurons · Glia or Supporting Cells · Neural Stem Cell (NSC) Culture · Glia (Astrocytes, Oligodendrocytes, Schwann Cells, Ependymocytes, Microglia) Cell Culture · PC-12 Cell Culture · HEK-293 Cell Culture

1 Introduction

The *nervous system* is a complex network of cells and their fibers called nerves that carry messages to and from the brain and spinal cord (The Center) to various body parts. While neurons are the typical cells within the nervous system that transmit the coordinated neuronal signals throughout the body, the glia cells are the specialized cells that support, protect, or nourish neurons and are, therefore, also termed supporting cells. In the nervous system, the number of glia cells is around ten times higher than the number of neurons.

The nervous system contains millions of neurons (~80 million as we are born with). As we grow, this number decreases. After attaining the age of 80 years, a human being may lose ~30% of neurons. Degeneration is the process through which neurons may degrade due to various reasons including damage or injury to the nerve

fibers. On the other hand, throughout our life, the nervous system can regenerate or repair the damaged neurons as well as form new nerve fibers, in a process called neurogenesis. Neurogenesis comprises the repairing of myelin sheath by oligodendrocytes and Schwann cells, within the brain and spinal cord, respectively (Sousa et al. 2017; Kettenmann et al. 1996).

In general, as a neuron progresses towards maturity, it loses the capacity to divide further. Neurons are unable to divide because they lack centrioles, the chief originators of cell division. So, the mature adult nerve cells do not divide by mitosis and are amitotic. Their DNA copying mechanism is blocked. However, new neurons come in place from neural stem cells (**NSCs**) during embryogenesis and are preserved even in adults within the specified brain regions such as the subventricular zone and other sections of the nervous system. Neural stem cells are presumed to play a decisive role in the manifested plasticity of an adult brain. It is now confirmed with sufficient evidence that neuronal stem cells (NSCs) in certain regions of the central nervous system (CNS), as well as other body parts, are capable of dividing further and can even migrate to other locations, as per the requirement (Imitola et al. 2004). As discussed above, this neuronal stem cell capacity to divide further is not restricted to embryonic stem cells and prevails even in adults (Clarke et al. 2000). So, the NSCs exhibit a therapeutic potential, particularly against various neurological degenerative diseases, and are therefore of immense interest to present-day researchers (Bonnamain et al. 2012). Experimentally, the generation of new neurons in adults was firstly demonstrated in birds, where labeled DNA precursors could be traced in differentiated neurons. Based on enormous experiments, it can be now conclusively claimed that neurons do quite more than just add up pulses: **In the decisive moments, they multiply** (Gordon et al. 2013).

Since the nervous system is very complex as well as compartmentalized amongst specialized environments, cell culture conditions of neurons derived from a specific brain region may differ from that of others. Moreover, since glia or supporting cells are a couple of times larger in number than neurons, it is very difficult to isolate and purify NSCs. One way to overcome the limited culture of NSCs is to establish the immortalized cell lines from the neuronal tumors. Since these immortalized cell lines would have unlimited growth capacity, a large number of cells would be generated in a very short time without any intercultural variability. However, since these are immortal cell lines, their physiology may be altered and therefore these cells may require different physiological culture conditions. For example, rat neuronal PC-12 cells (derived from adrenal medulla pheochromocytoma) cease to proliferate and undergo terminal differentiation into a neuronal phenotype on treatment with nerve growth factor.

Overall, two different neuronal cell models can be used in culture: Primary culture and culture of immortal cell lines. Neurons in primary culture are generally prepared directly from certain regions of the mammalian brain such as the hippocampus, cerebellum, striatum, etc. of the embryonic origin or other parts of the nervous system. However, the adult mammalian brain or other nervous system regions can also be used as they also contain NSCs. The cells isolated

from the above regions can be used to purify NSCs for further culture and propagation. After isolation and plating, every neuron, including those of mature adults, forms the synapses and becomes electrically active, acquiring a neuronal phenotype before ultimately perishing. However, as mentioned above, NSCs can divide as well as differentiate under specific cultural conditions and/or confluency. In contrast, immortal cell lines divide rapidly (log phase) on being plated and as they approach confluence, they begin to differentiate (Gordon et al. 2013).

As mentioned in the above paragraphs, while choosing an in vitro cell culture model based on primary cultures, one should consider the fact that neurons from different regions of the nervous system exhibit different characteristics. Some important brain regions from where the NSCs can be isolated for culturing include the cerebellum, the hippocampus, and the striatum.

For example, in the granular layer of the cerebellar cortex, cerebellar granule cells prevail as interneurons, while in the cerebellum, these represent the numerous neuronal cell populations. The purified cerebellar granular neurons are suitable for neuronal culture. Another example is embryonic rat hippocampal neurons. These cells are utilized for the study of neurite development, and synaptic transmission besides the functioning of voltage-sensitive and ligand-gated ion channels. Similarly, low-density striatal neuronal cells are used for morphometric and electrophysiological analyses. However, high-density striatal neuronal cells are used for biochemical and molecular analysis of neuronal cells. Additionally, as described in the previous paragraphs, several immortal neuronal cell lines have been developed. A major reason to establish these cell lines pertains to studying the effects of various compounds including drugs, toxins, etc. on the neuronal cells (Ray et al. 2014; Jana et al. 2007).

In this chapter, we describe the basics of isolation and primary culture of neuronal cells from various brain regions such as the hippocampus, striatum, and cerebellum. This chapter sheds light on the culturing methods of primary neuronal stem cells and various neurological cell lines such as PC-12 and HEK-293. Additionally, isolation and culture of glia or supporting cells, particularly microglial cell culture have been discussed.

2 The Nervous System

The *nervous system* is a complex network of cells and their fibers called nerves that carry messages to and from the brain and spinal cord (**The Center**) to various body parts. Structurally, the *nervous system* includes both the central nervous system **(CNS)** and peripheral nervous system **(PNS)**. While the CNS is made up of the **brain** and **spinal cord**, the PNS comprises the somatic and autonomic nerves that originate from the CNS. The PNS consists of **sensory neurons, ganglia** (clusters of neurons), and nerves, that are involved in neuronal connection including the CNS (Fig. 1).

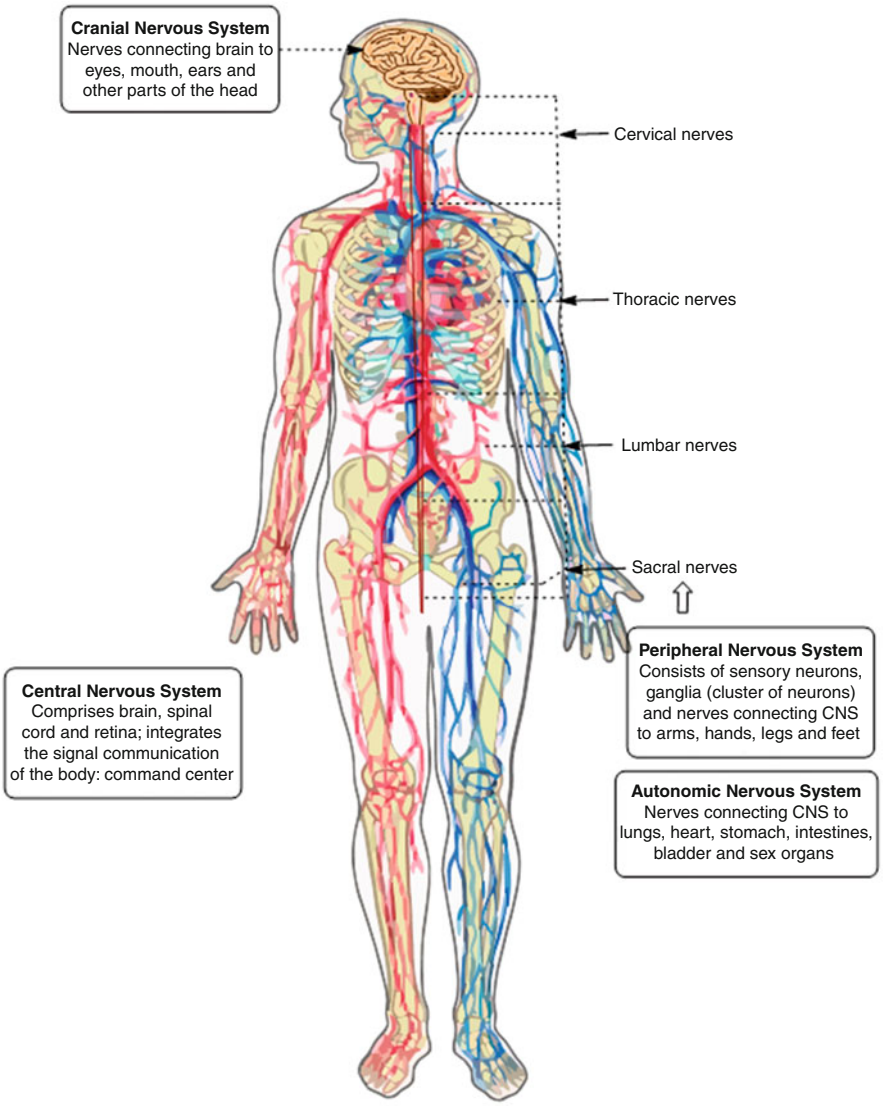

Fig. 1 Anatomy of human nervous system

Based on the functional variations, the nervous system is divided into two systems: The somatic or voluntary system and the autonomic or involuntary system. Through the somatic system, we control the signals originating from the brain and spinal cord (The Center) that are transferred to muscles and sensory receptors in the skin. In the case of the autonomic nervous system, various physiological functions such as heart rate, respiratory rate, blood pressure, etc. are involuntarily controlled.

The nervous system consists of two kinds of cells, which are **neurons** and **glia or supporting cells**. While neurons mediate the transmission of the coordinated neuronal signal throughout the body, glia cells are the specialized cells that support, protect, or nourish neurons. In the nervous system, glia cells prevail to nearly **ten times** greater intensity than neurons. There are extensive connections between the neuronal cells. When one neuron becomes excited and fires, it transfers the signal to the other neurons. Experimentally, it has been observed that one single neuronal cell can have up to 30,000 connections. These connections are between dendrons/dendrites and the axons/synaptic knob, the end terminals of the axon. While dendrons/dendrites receive the neuronal signals, the axons transfer the signals. In this case, larger the neuronal network, the better the chances of neuronal activation followed by a healthy survival.

2.1 The Neurons or Neuronal Cells

A neuron is the basic functional unit of the nervous system. Normally, a mammalian brain contains 100 million to 100 billion neurons, depending on the species. A typical neuron is a specialized conductor cell that receives and transmits electrochemical nerve impulses to other nerve cells, muscle, or gland cells. Neurons communicate with each other through an electrochemical process. When neurons receive or send messages, they transmit electrical impulses along their axons, which can range in length from a tiny fraction of an inch (or centimeter) to 3 feet (about 1 m) or even more.

2.1.1 Structure of a Neuron

A neuron comprises a structure further differentiated into a cell body, nucleus, dendrites, and the axon, as the main parts (Fig. 2). Dendrites and axons are responsible for accepting and transmitting neuronal signals, respectively, from one to another neuron. There are many connections between dendrites and axons, typically known as synapses. Thus, axons help to create networks whose function is to transmit messages from one to other neurons at the rate of 0.001 s, having a capability of occurring at about 500 times/s.

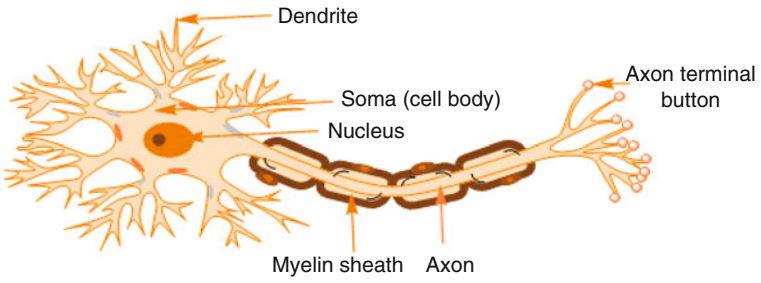

Fig. 2 Typical structure of a neuron: the messenger-coordinating unit of the brain

Here is a Brief Structure of a Neuron.

Cell Body
This is the part of the neuron that includes the nucleus and cytoplasm. It is in this space where most of the neuron molecules are synthesized or generated and the most important activities are carried out to maintain life and sustain a nerve cell functioning. A neuron cell body is also called Soma.

Nucleus
It is the central part of a neuron, having an oval-shaped structure located in the cell body. Its major function is to regulate the energy production, required for a cell's functioning.

Dendrons and Dendrites
Dendrites (the branches of a dendron) are the "arms of the neuron," and form branch extensions that come out of different neuron parts, mainly associated with soma or cell body. In other words, it is the minor extension of a cell body. The cell usually has many dendrites, the typical size of which depends on the neuron's function and where it is situated. Its main function is the reception of stimuli from other neurons.

Axon
In general, a single axon originates from the axon hillock region of the cell body and forms the largest extension of a neuronal cell. An axon extends from the cell body and often gives rise to many smaller branches before ending at nerve terminals. Each axon branch terminal is called a synaptic knob or synaptic button.

2.1.2 Synapse(s) and the Transfer of Nerve Signal

Synapses are the contact points where one synaptic knob or synaptic button of an axon comes in close contact with another neuron dendrite, having a very small gap between them called the **synaptic cleft**. The basic function of a synapse is to transfer a nerve signal from the synaptic knob of one neuron to the dendrite of another neuron. The neurotransmitters are stored in the synaptic buttons or knobs in small synaptic vesicles. As a neuronal signal reaches the synaptic knob membrane terminal (noted as the presynaptic membrane), the vesicles release neurotransmitters into the synaptic cleft, which subsequently diffuses across the dendrite membrane, called a postsynaptic membrane. This perturbs the membrane potential and activates the postsynaptic membrane to communicate a neuron signal.

Many axons are covered with a layer of the myelin sheath which, in turn, is made up of proteins and lipids. In the CNS, myelin is produced by oligodendrocytes, while in the PNS, it is produced by Schwann cells. The myelin sheath gives protection to the neuron and does not allow a transfer of ions across itself.

In certain axon regions, the myelin sheath is absent or broken, these regions are called **nodes of Ranvier** (Fig. 3). Since a nerve signal is not passaged through the myelin sheath but only through the nodes of Ranvier, it accelerates the transmission of nerve signals along the axon axis from one to the subsequent node of Ranvier. This kind of nerve signaling is called **saltatory conduction** because ions (such as

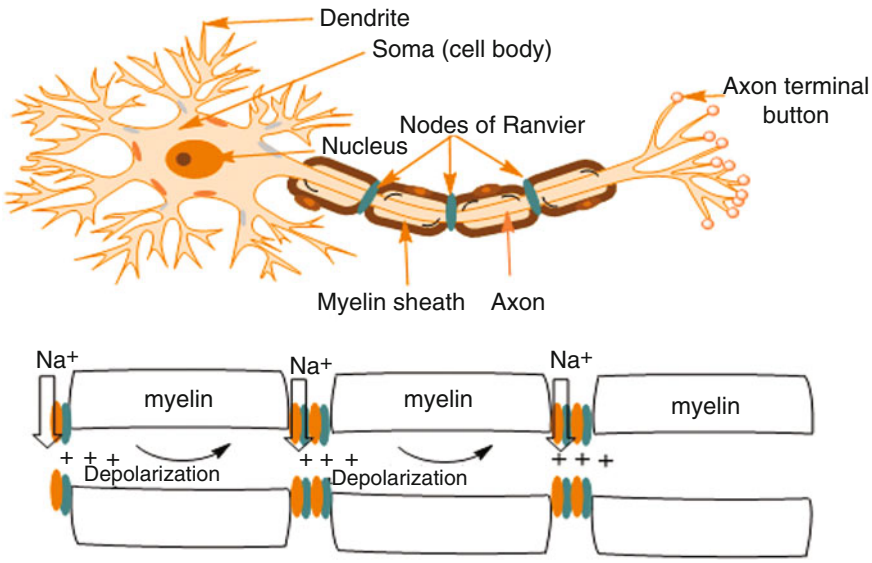

Fig. 3 Typical neuronal signaling of modulated (myelinated) nerve. Schematic illustrations of the myelin sheath, node of Ranvier, and saltatory conduction (lower figure), the coordinating neuron segments of an axon. The myelin layer forbids Na^+ and K^+ movement through the axon membrane. Depolarization and repolarization happen only in the gaps of the **myelin sheath**. These gaps are recognized as **nodes of Ranvier**. In the course of neuronal signaling, the impulse (Na^{++} K^+) jumps from one **node of Ranvier** to the next node. The typical sequence of events is termed **saltatory conduction**

Na^+ or K^+) jump or leap from one another node of Ranvier. This also enhances the speed of nerve impulses or nerve signaling. There are three major neuron types, typically, **sensory neurons** (for receiving a neuronal signal towards a nerve center), **motor neurons** (for communicating a neuronal signal from a nerve center), and **inter neurons** (communications between the neurons). All three have different functions, but the brain needs each one of them to communicate effectively with the rest of the body (and vice versa). On the other hand, based on the phenotype, neurons are divided into four types, namely, **unipolar, bipolar, multipolar,** and **pseudounipolar.** Physical damage to the brain and other parts of the CNS can kill or disable neurons. Likewise, the damage caused by a stroke can kill neurons outrightly or slowly starve them of the oxygen and nutrients they need for survival.

2.2 The Glia or Supporting Cells

The word **Glia** is derived from the Greek word for **glue**. These are the specialized cells that support, protect, or nourish the nerve cells. The brain contains around ten times more glia than neurons. In the brain, the glia is differentiated as per their specific functions. For instance, those making the myelin sheath are called **Oligodendrocytes**, and the ones coordinating PNS communications are known as

Schwann cells. Glia transport nutrients to neurons, clean up brain debris, digest parts of dead neurons, and help in holding up neurons in the right place.

The following are the types of glia observed in the mammalian nervous system.

2.2.1 Astrocytes

Astrocytes are star-shaped glia cells present in the CNS (Fig. 4). In humans, a single astrocyte cell can interact with up to **two million synapses** in an instant. The functions of astrocytes are quite diverse and include regulating the blood flow through the CNS, maintaining and regulating synapses, the places where neurons communicate with one another, and the storage and release of nutrition sources, especially during hypoglycemia. Thus, astrocytes are mainly responsible for **nourishing**, **cleaning,** and **supporting neurons**.

2.2.2 Oligodendrocytes

Oligodendrocytes are the large glial cells found in the CNS (Fig. 5). The vast majority of oligodendrocytes are involved in the formation of the myelin sheath, the protective cover of the CNS axons. Of note, the same function is performed by

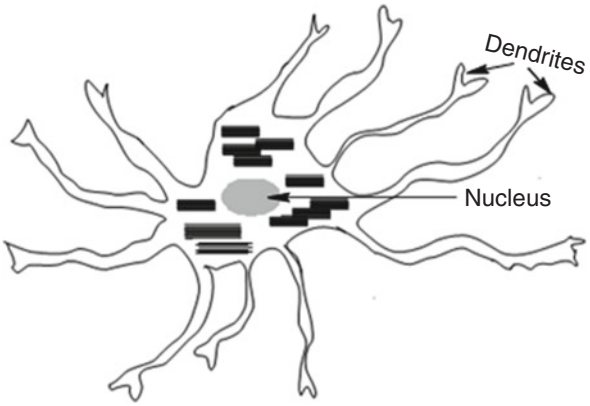

Fig. 4 Morphology of astrocytes; control units for neuron support, growth, development, and signaling coordination

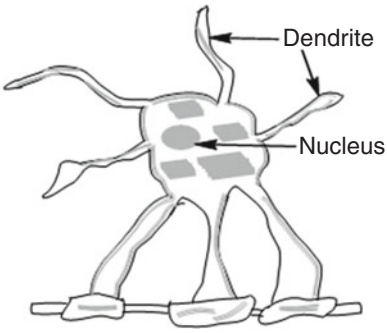

Fig. 5 Morphology of oligodendrocytes; control units for making myelin sheath and insulating axons from the concurrent Schwann cells

the Schwann cells in the PNS. However, a small number of oligodendrocytes called satellite oligodendrocytes are not involved in myelination. It is claimed that oligodendrocytes also aid in the functions of support and union.

2.2.3 Schwann Cells

The Schwann cells are a type of glial cells of the PNS which help in forming the myelin sheath around the nerve fibers. A typical Schwann cell envelops and rotates around the axon, forming a myelin sheath and resulting in the axon's myelination (Fig. 6).

2.2.4 Ependymocytes

The Ependymocytes or ependymal cells are the specific glial cells that form the epithelial lining of ventricular cavities of the brain and the central canal of the spinal cord (Fig. 7). The basic function of these cells is the protection of the above-mentioned areas of the nervous system.

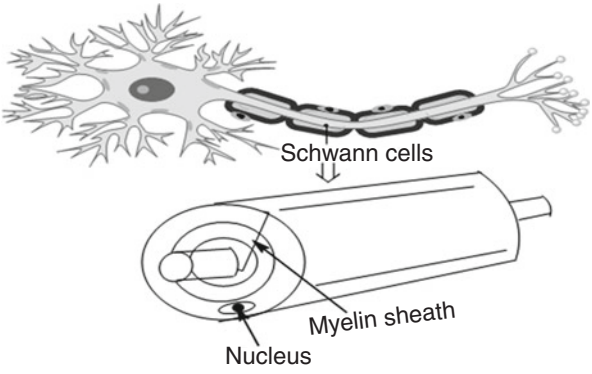

Fig. 6 Segmented enlarged morphology of Schwann cells; the functional units of myelin sheath surrounded axons

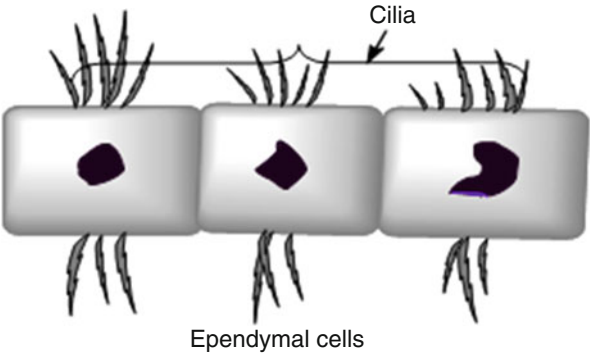

Fig. 7 Integrated assembly of ependymal cells, forming the epithelial layer of the brain and the central spinal cord canal

2.2.5 Microglia Cells

Around **20%** of the total glial population is occupied by microglial cells. These cells are described as **immunological cells of the CNS** (Fig. 8). Based on their specific origin, the microglial cells are resident macrophages originating from the yolk sac. These cells repopulate the CNS parenchyma during early development and are self-renewed locally. Experimental results demonstrate that microglial cells are independent of bone marrow-derived monocytes. The endothelial cells present in the CNS act as a barrier to forbid the transfer of most of the molecules in the brain, eminently recognized as the Blood-Brain Barrier (**BBB**). Therefore, the CNS is considered an **immune-privileged organ**.

Experimentally, it has been observed that the microglial cells exhibit highly sensitive potassium channels, which respond to minor changes in potassium extents within the CNS. The microbial infections in the CNS may lead to alteration in the potassium channel. Under these prevailing conditions, the microglial cells perform a crucial role by conferring immunological protection to the CNS.

3 The Division of Neuronal Cells

Mature adult neurons cannot divide as they **lack centrioles**, the main architects of chromosomal movement during cell division. So nerve cells do not divide by mitosis and are amitotic. Thus, their DNA-copying mechanism is blocked. However, over time, new neurons come from NSCs that are predominantly preserved in the subventricular zone during development. The NSCs are presumed to play a key role in regulating the plasticity of an adult brain. So, these cells exhibit a therapeutic potential, particularly against various neurological disorders.

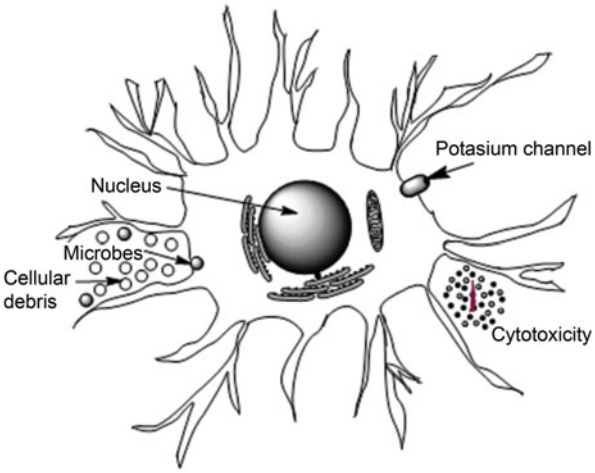

Fig. 8 Typical morphology of microglia cells, control units of immune defense, waste elimination (from neurons), and homeostasis

New neuron generation was first described in birds. Later, experiments revealed that new neurons can also be developed in adult mammalian CNS, including human CNS. However, this generation of new neurons was noticed not in the whole brain but only in some restricted parts. For example, the **granular cell layer** and **dentate gyrus** of the **hippocampus** are the major regions of the brain which contain the NSCs that give rise to new neuronal cells. These new neurons are local circuit neurons and interneurons, not long-distance neurons. These brain cells can migrate to the brain areas that need it most, allowing for a partial restoration of the damaged area. The process not only allows the brain to communicate the information much faster than presumed but also regulates a multiplication of single neurons, opening the door to more complex forms of computing. It also became evident subsequently that neurons do more than just add up pulses, getting multiplied in the decisive moments. While NSCs can be preferentially isolated and cultured from developing embryos, they can also be isolated even from the adult nervous system (Sousa et al. 2017; Kettenmann et al. 1996).

4 The Culture of Neuronal Cells

- Primary neuronal cultures are indispensable in the neurobiological and pharmacological fields.
- ***The primary cultured neurons are used to study morphology, electrophysiology, neurotoxicity, neurotransmitter release, synaptic function, and disease modeling***.
- *For these investigations* ***microscopy, single-cell or multisite electrophysiological recordings, for example, patch clamp, Micro-Electrodes Arrays (MEAs), and calcium imaging*** *are utilized*.

Yet the primary culture of neurons is notoriously difficult because of the following reasons:

- In general, cultured neurons have a short survival time.
- Cultured neuronal cells are highly susceptible to infection.
- Cultured neuronal cells are sensitive to hyperosmolarity arising from medium evaporation.
- Growing and maintaining primary neurons for long durations in culture requires excellent cell-culture handling skills of the experimentalists along with an optimal combination of media, serum-free supplements, and substrates. Paying close attention to these culture conditions is important to ensure the ones required for primary neurons' survival.
- Additionally, adequate success along with righteous structural-functional features of cultured neuronal networks depends on the following factors:
  - The animal model being studied.
  - The origin of the tissue being analyzed.
  - The age of the culture.

  - The cell density.
  - The physical and biochemical environment.
  - The optimum contributions of the above features evolve as manifestations of neuronal network differentiation and maturation.
- ***Age of the culture plays a very important role in the electrical activity of the neurons.*** For example, the electrical activity on the **7th day** of culture is characterized by only a single spike, while on the **14th day**, culture networks exhibit an enhanced firing rate with episodes of high-frequency spiking, a rich and stable burst pattern. This highly synchronized instant of high-frequency activity is accompanied by simultaneously encompassed manifold network sites.
- Additionally, several studies have revealed the effect of **neuronal cell densities** on the functional properties of developing neuronal networks. It is documented that the morphology of the dendrites and the synaptic density is influenced by the density of the neurons leading to variations in cell to cell contacts. Several experimental works have been completed in this research area. Based on the experimental results, it was claimed that ***sparse networks exhibited stronger synaptic connections between pairs of recorded neurons compared to dense cultures***. ***Additionally, in sparse cultures, the spontaneous network activity with enhanced burst size was noticed contrary to the reduced burst frequency and a less synchronized activity in the dense cultures.*** It has been observed that cell density has an important role in the maturation of cultured neurons since densely seeded cultured neurons mature faster than sparsely seeded neurons. However, culture-to-culture variability is always an important factor. In this case, the optimal plating density of cerebellar granule cells is 500–800 cells μl^{-1}. At this plating density, the cell survival rate is very high.
- The **developmental stage of the animal** is another important factor that needs to be considered while isolating neuronal cells for culture purposes. For example, **6–8 postnatal days** is the optimal duration for granule cell culture since this time represents the characteristic developmental availability of these cells. Similarly, it is claimed that **gestational 18th day through postnatal day 1** should be chosen for the isolation of Purkinje cells, as this is the time when the granule cell population is yet to develop. Finally, during the isolation of neuronal cells for culturing, surrounding tissue must be thoroughly eliminated to minimize the unwarranted neuronal cells, interference (Biffi et al. 2013).

Regarding proper isolation and culture conditions, below aspects are important:

4.1 Regarding the Use of Proteolytic Enzymes

The proteolytic enzyme, trypsin is the most common enzyme used for the dissociation of most tissues including neuronal tissues. However, another proteolytic enzyme, that is, papain is also frequently used in the isolation of intact/undamaged neuronal cells for culture purposes. Of note, papain exerts much lower damaging

effects on the neuronal cells than trypsin. Thus, papain is used both for fetal and postnatal brain tissue to isolate the maximum number of viable neuronal cells.

The following aspects should be considered amidst preparing a cell suspension of maximum purity:

- The proteolytic enzymes should be highly pure.
- Triturating should be gentle.
- Cell manipulation time should be kept to a minimum.
- The plating density should be proper.

NB: ***It is generally recommended that neuronal cells are highly sensitive and contain extensive branching of nerve fibers; therefore, mild handling and washing with a complete cell culture medium instead of PBS or DPBS or HBSS is necessary to prevent unnecessary cell death. After plating the neurons, they are left undisturbed at room temperature away from equipment vibration for ~30 min to adjust to their environment. Any sort of shaking or movement of the cell culture containers can prevent neurons from long-term growth, causing variability in the culture data. Additionally, the exact protocol concentration of the proteolytic enzymes must be maintained as an unusually high concentration may be detrimental to cell growth.***

4.2 Regarding the Use of Deoxyribonuclease 1

During proteolytic treatment and cell isolation, the damaged or dead cells may release DNA that may interfere with the cell isolation. In this case, DNase 1 is used to break down the DNA. Since DNase 1 is activated in presence of bivalent metals, it is necessary to add suitable sources thereof (such as $MgCl_2$) during cell isolation.

4.3 Regarding the Use of Cell Adhesive Agents and Culture Containers

For biochemical methods, cells are plated directly into the wells of a **Poly-L-lysine-** or Poly-D-Lysine-coated culture dish. Other adhesive agents such as collagen, laminin, etc. can also be used. Some studies claim that a better adherence could be achieved with the poly-D-lysine/fibronectin or poly-D-lysine/laminin coating. Poly-ornithine, poly-D-lysine, and laminin, when used in combination with a serum-free basal medium and serum-free supplement, improve the overall growth and performance of primary neurons. However, a double coating is expensive and needs 1–2 days for completion. Poly-L-lysine and laminin-coated dishes are also used for culturing neural precursor cells. When using poly-L-lysine, a formulation with a higher molecular weight is recommended since the shorter the polymer, the more toxic it can be to the neurons, possibly detaching them. In several studies, matrigel has been used as a substrate with various applications. In a few reports, collagen has

been used as an attachment agent. For histochemical staining and live imaging, the cells are grown on glass coverslips. For biochemical analysis, cells must be grown in containers of different sizes. Additionally, for biochemical assays, such as transcriptomic and proteomic analyses, it is essential to minimize the number of other cells in the culture, particularly the number of glial cells.

NB: ***Typically, a few reports noted the clumping of neuronal cells in 96-well plates, not in large cell culture containers. The reasons may be related to the drying of the coating matrix before seeding the cells.***

4.4 Regarding the Use of Serum-Free or Serum-Supplemented Medium

Neuronal cells can be cultured in both, serum-free and serum-supplemented media. While serum may boost the growth of neuronal cells, its use can also have certain ill effects on cultured neuronal cells. For example, the serum contains growth factors, hormones, cytokines, and other molecules that affect the growth of the neuronal cells. Additionally, in the serum-supplemented medium, it is difficult to control the growth feeder glial cells. On the other hand, the use of a serum-free medium for the neuronal cell culture may have several added benefits. For example, it allows the cells to grow homogenously. The serum-free medium facilitates the culture of low-density neurons, including the study of individual neurons and synapses.

4.5 Regarding the Use of Various Growth Supplements

The neuronal cells require various growth supplements for their proper growth in culture. The critical growth supplements for the neuronal cell culture **are growth factors, hormones, amino acids such as L-glutamine**, etc. While glutamine is an essential amino acid needed for the culture of various cells including neuronal cells, the degradation of glutamine produces ammonia and carboxylic acid which are toxic to the cells. So, glutamine may be replaced with **glutamax** (Invitrogen), which does not have any stringent toxic effects. In normal physiological conditions of an intact brain, these growth supplements are supported by glial cells, and therefore their name as supporting cells justifies. Perhaps, this is the reason for adding a feeder layer of supporting glial cells during the neuronal cell culture. Unfortunately, as the glial cells grow, these cells produce toxic metabolites that could severely affect the neuronal cell growth in the culture. Thus, as indicated in the previous paragraphs, a serum-free medium is the best option for culturing neuronal cells. The serum-free medium can also be utilized to examine the effects of growth factors, and hormones on the neuronal cells. In general, the much-used serum-free medium is a neurobasal medium supplemented with B27. While B27 is optimized for the growth of the hippocampal neurons, other neurons also grow well in presence of these supplements.

Growth factors are as much important in a culture medium as that in the substrate. For example, to undergo neuronal differentiation, the neural cell line (**PC-12 cells**) is treated with neuronal growth factor **(NGF)** in the absence of serum. For the culture of neuronal progenitor cells, epidermal growth factor **(EGF),** basic fibroblast growth factor **(bFGF),** and insulin-like growth factor-1 **(IGF-1)** are generally added to the cell culture medium. bFGF is also added to the culture medium for the growth of multipotent neuroepithelial **(NEPs)** cells. **Collectively, the age of the cultured cells, density of the cells, and the type and amount of growth supplements including various growth factors, all comprise important ingredients for optimum neuronal cell culture.** Thus, morphology, differentiation, adhesion, and growth are greatly affected by the specific growth of substrate used while culturing the cells.

4.6 Regarding Detachment of Cultured Neuronal Cells

For the detachment of neuronal cells from the cell culture containers, **collagenase** can be used if the attaching agent is collagen. Several reports used **trypsin or papain** and a few reports also used **accutase**, particularly when neuronal cells are used for **FACS analysis**.

5 Neuronal Cell Culture Isolated from Hippocampus

Hippocampus is recognized as a part of the limbic system, a cortical region of the brain. It is anatomically located deep inside each temporal lobe of the cerebral cortex. The **dentate gyrus, CA3, CA2, CA1, subiculum**, and **entorhinal cortex** are the structural constituents of the hippocampus (Fig. 9). Hippocampus is connected through both afferent and efferent neurons. The cingulate gyrus, dentate

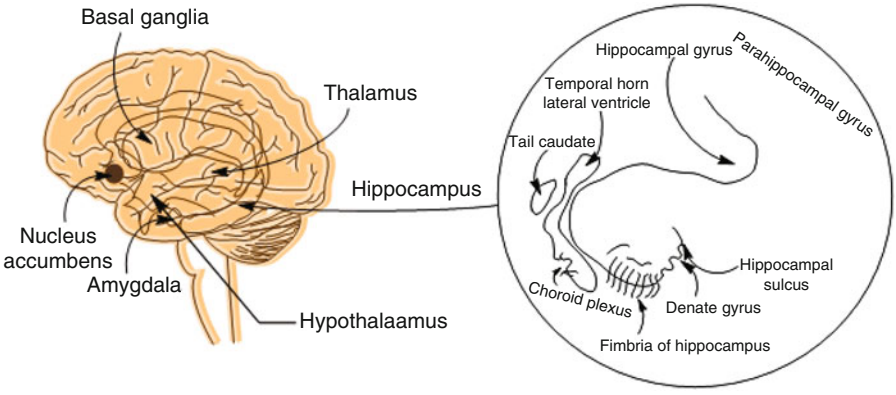

Fig. 9 Typical segmented view of the hippocampus, regulating unit of memory encoding, consolidation, and responsive coordination. This compartment is readily preferred for brain cell culturing and contains the richest population of neural stem cells

gyrus, contralateral hippocampus, parahippocampal gyrus, septal area, indusium griseum, and diencephalon send afferent signals to the hippocampus. On the other hand, the subiculum to the entorhinal cortex and amygdala, as well as through the fornix to various anterior brain domains, are used to send efferent signals from the hippocamps.

Hippocampus is involved in the following functions:

- As a part of the limbic system, the hippocampus is involved in several fascinating functions of the brain. Briefly, the most prominent function of the hippocampus is the activation and regulation potentiation of **long-term potentiation (LTP)**, a candidate mechanism for memory. Thus, the hippocampus performs a key role in memory consolidation and spatial navigation. Besides memory, the learning capacity of an individual is controlled by the hippocampus.
- Besides learning and memory, the hippocampus is also involved in regulating goal-directed behavior.
- Several behavioral hippocampus characteristics include behavioral inhibition, obsession analytics, and scanning and spatial map formation.
- The most striking feature of the hippocampus function is that **it does not have a regulation towards controlling behavior in response to the characterization of an experience**. Additionally, it has been experimentally documented that external stress-induced enhanced corticosterone level eventually reduces the firing rate of the hippocampus.
- Some of the increased sensory responses of the cerebral cortex such as vision, hearing, and touch are influenced by the low-frequency firing response of the hippocampus.

5.1 Types of Neuronal Cells Present in the Hippocampus

The hippocampus contains three types of cells, namely, pyramidal cells, granule cells, and interneurons. The approximate number of the pyramidal and granular cells in the hippocampus is known. For pyramidal cell numbers, the CA3 and CA2 domains, CAI field, and subiculum contain 330,000, 420,000, and 128,000 cells, respectively. On the other hand, the dentate gyrus contains 1,000,000 cells. Thus, pyramidal cells are the major cells in the hippocampus. However, while interneurons are very few*, their exact number in the hippocampus is not known.* Figure 10 pinpoints the structural distinctions of pyramidal neurons and interneurons.

Ahead is a brief discussion of the cell types present in the hippocampus.

5.1.1 Pyramidal Neurons

Nearly ninety percent of the total number of hippocampus neurons are occupied by the pyramidal cells, which are located in the CA1 and CA3 regions. The pyramidal neurons exhibit a well-defined triangular cell body that looks like a pyramid. Hence, the pyramidal nomenclature is justified. Each pyramidal cell contains one single large axon. These cells are not only interconnected with other neurons but also with

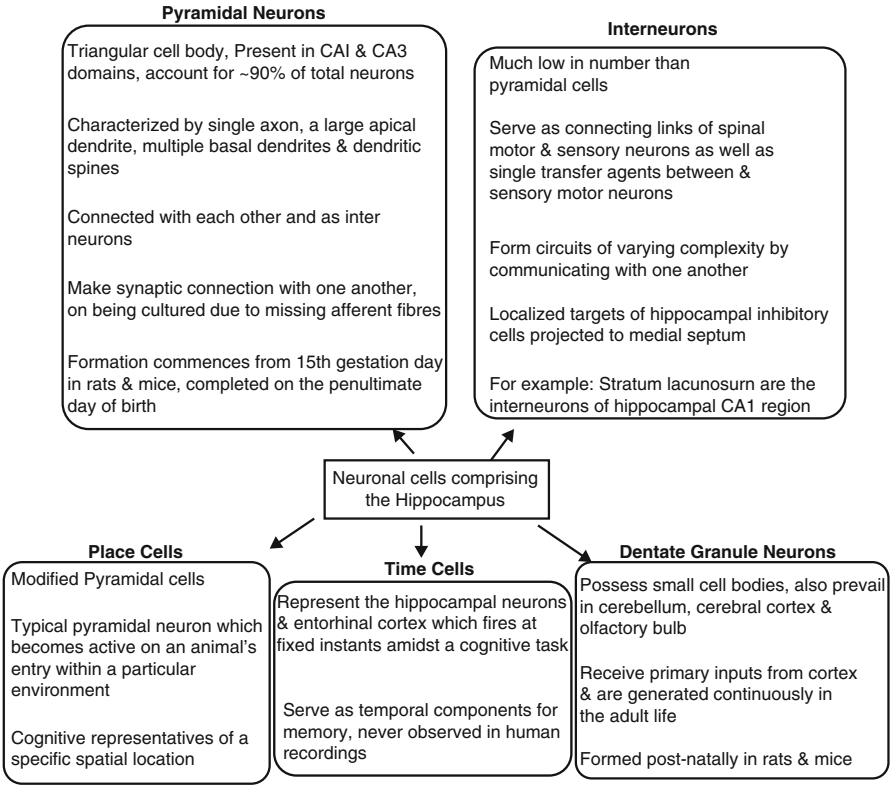

Fig. 10 The functional distinctions of constituent neuron cells within a hippocampus

other pyramidal cells. Broadly, pyramidal neurons constituted two kinds of cells, that is, **Place cells** and **Time cells**.

Here is a very brief discussion about them.

Place Cells

These cells represent a kind of modified pyramidal cells. These specialized pyramidal cells become activated when an animal enters a specific place. Thus, this nomenclature is justified. Based on the specific location or environment of the location, these cells activate the cognitive function and develop a cognitive map of the animal.

Time Cells

These specialized neuronal cells are present in the hippocampus and entorhinal cortex. The cells are so named because of their ability to place a sort of **time stamp on memories** as they are being formed. This type of time stamp is highly essential to recall sequences of events or experiences in the right order.

5.1.2 Interneurons

The presence of several interneurons has been reported inside the hippocampus. The interneurons are less in number as compared to the pyramidal cells. For example, in the hippocampal CA1 region, the interneurons present are called **Stratum lacunosum molecules**. These neurons can communicate with each other and are connected in transferring sensory and motor signals linking the spinal cord. It is claimed that the interneurons are the local targets of hippocampal inhibitory cells that project to the medial septum.

5.1.3 Dentate Granule Neurons

The third type of neuron (granule neurons) is the dentate gyrus. These cells have small cell bodies. Besides the hippocampus, these cells are present in various regions of the brain, including the cerebellum, cerebral cortex, and olfactory bulb. The hippocampal granule neurons receive signals from the cortex. Throughout a human being's life span, the hippocampal granule neurons keep on generating new neuronal cells.

The primary culture of hippocampal neurons may help one to understand the brain's ability to learn and remember. As indicated in the previous paragraphs, before beginning the hippocampal culture from mice and rats, one should know that pyramidal neurons begin from the 15th gestational day and cease in growth before the day of birth. On the other hand, the generation of dentate granule cells occurs postnatally. This implies that the time of **pre- or postnatal** hippocampal cell isolation and preparation can determine a culture's neuronal composition.

5.2 The Reasons for Choosing Hippocampus Cells for Culture

Of all the various brain regions, the hippocampus is the most widely used for the isolation and culture of neuronal cells because of the following reasons:

- The **simple architecture of the hippocampus** with the availability of the division/proliferation-capable cells makes the hippocampus an ideal organ to isolate and culture neuronal cells.
- In general, late-stage embryonic tissues are preferred for the culture due to the less number of glial cells than isolating cells from the mature brain tissues.
- Additionally, the embryonic hippocampal tissues have fewer adhesion molecules compared to the adult cells and are therefore easy to isolate. While both mice (E19) and rats' embryonic tissue can be used for the isolation of the hippocampus, the mouse hippocampus is difficult to isolate because of its small size. Following separation of the hippocampus, the cells may be dissociated easily by mild treatment with proteolytic enzymes (e.g., trypsin/papain) or mechanical disaggregation.
- Hippocampus is a rich source of NSCs, which can divide, as well as differentiate in appropriate conditions along with a potential to migrate to other regions of the brain.

- Once neurons are isolated and cultured, advanced molecular techniques for screening their subcellular localization and trafficking can be employed.

5.3 Isolation and Primary Culture of Neuronal Cells from Hippocampus of Prenatal Mouse

The protocol described in the following paragraphs originated from the publication of Seibenhener and Wooten (2012) with modifications.

- In this procedure, mouse and prenatal embryos were used as the sources of hippocampal cells.
- Since prenatal embryo was used, the contaminated glial cells were none to very less.
- The cell culture medium utilized for this culture is a neurobasal medium supplemented with B27, in a serum-free environment.
- No feeder cell layer was utilized.
- The harvested hippocampus was treated with diluted trypsin for 3–5 min. However, papain is also very useful for the isolation of cells.
- Following cell dissociation, tissue triturating was completed using two sterile Pasteur pipettes. Please note that the "rough" treatment at this stage could be detrimental to neuronal cell survival. Trypsinized tissue should be passaged through the pipette at a consistent flow rate. Alternatively, using a syringe fitted with 22 gauge needle trituration can also be done. Of note, this is the most sensitive step of isolating the intact live cells.
- Following cell isolation, trypan blue staining is completed to identify the dead cells. Generally, at stage 70–80% of the isolated cells are viable. Since seeding resulted in ~20% of the cells being perished on overnight incubation, it is preferable to use a 20% higher seeding density to compensate for the dead cells. This is necessary because cells seeded in a very low density may not grow at all.
- The fresh medium should be supplemented every 4 days interval.
- It is observed that neuronal growth under these conditions can be easily carried out for 10–14 days in culture.
- Further, the culture has been used to propagate neurons for 30 days on being seeded at 80 cells mm^{-2}.

5.3.1 Steps for Isolation and Primary Culture of Neuronal Cells from Prenatal Mouse Hippocampus

Step 1: Breeding Schedules to Collect Prenatal/Postnatal Pups

C57BL/6 mice ages 2–8 months were used in mating to develop this protocol.

- Schedule breeding between adult mice 19 days before the day of neuron isolation to generate prenatal pups for neuron harvest.

NB: Confirm mating either by observing visual pregnancy or* via *detection of a vaginal plug in the female palpitation.

- ***Typically, one can find most protocols using E18-E18.5 pups for this experiment, as one gets purer cultures (astrocytes will start kicking from P_{20} and so on)***.
- However, in the hippocampus neuronal culture protocol, the ***rat fetuses obtained at the 20th gestational day can also be used***. In this case, the culture consists majorly of pyramidal cells.
- 0.5-day-old-pups can be used to generate hippocampal granule cells.

Step 2: Preparation Before the Day of Embryo Harvesting

Collect Sterile Dry Instruments for Surgery, Cell Isolation, and Culture

- Use either pre-sterile instruments or make them sterile, 1 day before surgery for every dissecting instrument such as dissecting scissors, forceps, syringes, etc. Keep the sterile and dried instruments ready for surgery on the next day.
- Other sterile materials which are necessary for dissections and cell culture include glass pipettes, 5, 10, and 25 ml sterile disposable pipettes, 0.2 μm syringe filter, syringe: 5 cc, centrifuge tubes, 15 ml, sterile Pasteur glass pipettes, sterile cell culture vessels, glass cover slips, and dissecting microscopes.

Autoclave Double Distilled Water and Dissecting Solution

Autoclave double distilled water (ddH_2O) and dissecting solution (DS).

Preparation of Complete Cell Culture Medium

The serum-free neurobasal medium in a combination of B27 (**Gibco**) or N-2 is used for long-term maintenance and maturation of pure pre-natal and embryonic neuronal cells. This medium composition does not need to use astrocytes as feeder cell culture. However, in the serum-containing medium of 0.5 mM, L-glutamine or GlutaMAX™-I Supplement may be used. For initial plating, 25 μM glutamic acid is required to be included. No L-glutamine, L-glutamic acid, or aspartic acid is needed, aliquots and stored at 40 °C.

Preparation of Poly-D-Lysine and Coating of the Cell Culture Vessels

- 70–150 K Poly-D-Lysine (PDL) from Sigma (5 mg) was dissolved in 10 ml sterile H_2O overnight on a rotary shaker on being treated at 50–100 rpm, depending on the orbit diameter. Aliquot and freeze at −20 °C.
- Dilute the above 10× PDL to 1× PDL by adding 9 ml sterile ultrafiltered water/PBS to 1 ml of 10× PDL stock and mix well (1×). This 1× PDL in PBS is used for coating the culture vessels overnight at 37 °C. Generally, one can use as high as 50–70 μg ml^{-1} PDF as the final concentration.
- Other possible coating materials include laminin, fibronectin, and collagen. They can be used in combination with PDL (e.g., several authors used collagen 1 from rat tail and PDL in 3:1 proportion) or alone, but the latter option is generally not suitable for mid- to long-term CNS neurons culture and is rather more suited for the PNS.

- Rest the plates uncovered in a tissue culture hood overnight. This hood must be equipped with a UV light irradiation facility.

Step 3: Embryos/Pups Preharvesting on the Day of Harvesting

- Wash the dishes or glass coverslip with sterile HBSS after incubation with coating agents.
- Since water is toxic to the neuronal cells, it is recommended not to use water to wash the plates.
- The excess plates must be stored at 4 °C in the dark (i.e., in presence of HBSS), to prevent them from drying.
- **In general, the coated but dried culture vessels are not recommended for use.**
- Place the proteolytic enzymes (e.g., papain and the DNase stock solutions) in the water bath until needed.
- Warm the cell culture medium and get it ready for cell harvesting and culture.
- Minimize the time, making sure that all needed materials (e.g., coated culture vessels, proteolytic enzymes, and cell culture medium) are ready before starting the procedure. The dissection usually lasts ~2.5 h.

Step 4: Isolation of Mouse Hippocampus

- Generally, it is recommended to complete every single step inside a laminar flow hood.
- Decapitate a pregnant mouse ~19 days after fertilization.

  NB: Euthanization of the pregnant female mouse is not recommended since anesthesia causes brain death (Stratmann et al. 2014). The scissors, forceps, and all other small instruments necessary for the isolation of the hippocampus must be sterile.
- Wipe the abdomen of the mouse with 70% ethanol.
- Remove the skin.
- Cut the abdominal wall and open the abdomen.
- Remove the uterus and put it into a sterile 100 mm Petri plate.
- Now open the uterus and remove the pups.
- Decapitate the pups under a dissecting microscope.
- Open pup cranium from the back of the neck to the nose using sterile scissors or a scalpel. This procedure can be completed normally via inserting one scissor tip into the vertebral foremen and then proceeding via the anterior route.
- Using forceps, carefully isolate the whole brain.
- Place the brain on sterile gauze.
- Remove the cerebellum using a sterile scalpel and incise down the midline to retrieve the two hemispheres.
- Grasp a small section of meninges surrounding the hippocampus with sterile forceps and gently pull it away. The hippocampus becomes more clearly visible after removing the meninges.
- The hippocampus is a curved structure that starts in the distal part of the hemisphere and bends ventrally. As the inner, concave side (caudal) faces a ventricle, it is already free, it is, therefore, alright to isolate the hippocampus for which one needs to cut along the outer convex edge.

- Collect the hypothalamus in a 35 mm Petri dish filled with CMF-HBSS, 37 °C.
- Separate the two cerebral hemispheres from the brainstem.
- At this stage, if the thalamus is still attached to the hippocampus, gently remove it using fine forceps.

NB: Brain tissues can be combined from multiple pups, depending on the number of cells needed or the culture vessels needed to be grown.

Step 5: Dissociation of Neuronal Cells from the Isolated Hippocampus

- Using a sterile scalpel, gently mince brain tissue in 3 ml sterile HBSS, in a 100 mm tissue culture dish.
- Take a 15 ml Falcon tube, and transfer the minced tissue to the HBSS solution.
- Remove excess HBSS solution after a few minutes.
- In 3 ml minced tissue HBSS, add additional 1.5 ml HBSS and 0.5 ml 0.25% trypsin. Now the total volume is 5 ml.
- Alternatively, the following materials may be added to the minced tissues: papain, DNase stock solution (1:100 dilution), and 5 mM $MgCl_2$ (final concentration).
- Close the cap of the 15 ml tube.
- Slowly mix the contents by inverting the tube a couple of times without forming any air bubbles.
- Tissue may float if air bubbles are formed.
- Incubate hippocampal tissue at 37 °C for 15 min, inverting tube as above every 5 min.
- Allow the tissue to settle at the bottom.
- Wash the tissue pellet with 5 ml HBSS at 37 °C for 5 min.
- Again allow the tissue to completely settle at the tube bottom each time.
- Remove the final wash from the tissue pellet and replace it with 2 ml fresh HBSS.

Step 6: Neuron Trituration

- Make fire-polished Pasteur pipettes.
- Using a normal sterile 9-inch Pasteur pipette, gently triturate the tissue for seven times.
- Larger tissues should settle at the bottom of the tube.
- The supernatant should be transferred to a fresh sterile 50 ml conical tube.
- Add 2 ml sterile HBSS to the remaining tissue and triturate five times using the fire-polished Pasteur pipette.
- At this stage, allow all remaining larger tissue pieces to settle to the tube bottom.
- All the supernatants should be combined.
- Centrifuge at 300× g for 3 min at 4 °C.
- Decant the supernatant and wash the hippocampal tissue once with CMF-HBSS.

Step 7: Cell Plating and Culture

- Use a hemocytometer to count the cells.
- Plate the cells according to the following recommendation:
  - For coverslips in a 24-well plate: 6×10^4 cells/well per 0.5 ml
  - For 60 mm tissue culture plates: 4×10^5 cells/plate per 3 ml
  - For 100 mm tissue culture plates: 6×10^6 cells/plate per 6 ml

- Mix appropriate cell numbers with indicated Neurobasal Plating Media (Neurobasal media containing B27 supplement [1 ml 50 ml^{-1}], 0.5 mM glutamine solution, 25 μM glutamate (Mr 147.13 g mol^{-1}) volume of penicillin (10,000 units ml^{-1})/streptomycin (10,000 μg ml^{-1}) [250 μl 50 ml^{-1}], 1 mM HEPES (Mr 238.3 g mol^{-1}), 10% heat-inactivated Donor Horse Serum), and add cells to plates.
- Gently swirl the cell culture containers to evenly distribute the cells.
- In this research protocol, the HI-Donor Horse Serum was added to the plating media to enrich the cell growth during the initial 24 h.
- Incubate the cells in a 37 °C, 5% CO_2 incubator overnight.
- Following 24 h of incubation with the serum, the culture medium was gradually replaced with the serum-free medium. The low concentration of glutamine used in this is likely to inhibit the attachment of nonneuronal cells. Like serum removal, it is also removed from the culture medium after 24 h since a high glutamine concentration is toxic to the neuronal cells.
- Remove half of the medium volume from the cells and replace it with an equal quantity of **Neurobasal Feeding Medium** (Neurobasal Media containing B27 Supplement [1 ml 50 ml^{-1}], penicillin (10,000 units ml^{-1})/streptomycin (10,000 μg ml^{-1}) [250 μl 50 ml^{-1}], 1 mM HEPES (Mr 238.3 g mol^{-1})).
- Every 4 days replace the old medium with the fresh Neurobasal Feeding medium.
- It is predicted that neuronal developmental events ideally become noticeable on day 1 and prevalent from day 10 onwards.

5.4 Cautionary Points During Cell Culture Originated from the Hippocampus

- One of the most fundamental methodologies for modern neurobiology is the primary neuron culture from the embryonic rodent hippocampus or cortex. While initial studies used serum-containing medium, gradually defined serum-free media gained preference. Experiments on neural cell culture demonstrated that just like any other cell culture, modifying the culture medium composition and conditions affects the survival, growth, differentiation, apoptosis, and even the overall phenotype of the cells. The protocols described by various researchers claimed that neurons could be easily cultured over a few days or weeks and these cultured neuronal cells become capable of differentiating into clearly distinguishable axons, dendrites, dendritic spines, and synapses.
- In general, seeding the isolated cells for the first time with the serum-containing medium settles them (at once), resulting in their attachment to the culture vessels. The serum is gradually removed from the culture medium at a decreasing concentration. Readers must note here that sudden removal of the entire serum at one go may kill the neuronal cells via nutritional shock(s) and other adversities.
- While culturing certain neuronal cells does mandate the presence of a limited number of glia cells as a feeder layer that supports their growth, some other research protocols treat the glia cells' presence as a contaminant. Therefore, these use procedures to remove the glia cells from the neuronal cell culture. It must be noted hereby that glia cells have specific and distinct morphology that differs

from neuronal cells and aids in their explicit identification. In some experiments, the glia cell contaminations do not affect the documentation of the experiments. For example, in the immunofluorescence experiments, the glia contamination can be treated as a rather inconvenience while photographing the individual cells.

- Several investigations have reported that a minor extent of glial cell contamination may aid the hippocampus-originated neuronal cells to grow better. This is based on the belief that feeder glial cells are supporting cells that secrete several materials to help in the growth, survival, maturation, and plasticity of cultured neurons. In a parallel trend, several reports indicate that aggravated toxicity of a high number of feeder glia cells may be toxic to the cultured neuronal cells due to a release of various toxic metabolites.
- In the experimental protocols, where glial cell contamination is not warranted, initially, cultured cells may be treated with **cytosine arabinoside (AraC)** at **5 μM** for **3–4 days**. **The higher concentration of this drug or a long duration of treatment may be toxic to the cultured neuronal cells.** Some research protocols use **5-fluoro-2′-deoxyuridine (FUdR)** to decrease the proportion of fibroblastic-reactive microglial cells.
- It is noted that reproducing neuronal cell culture protocols is sometimes highly difficult on many occasions, in particular, those being exercised in laboratories different from their standardized ones, due to variations in respective cell source, age of derivation, and culture conditions (Sahu et al. 2019; Brewer et al. 1993; Rybachuk et al. 2019).

6 Isolation and Primary Culture of Neuronal Cells from Striatum

The striatum is a critical component of the motor and reward systems, the impaired functioning of striatal neurons can lead to multiple neuronal disorders, from obsessive-compulsive-like behaviors to neurodegeneration, as witnessed in Huntington's disease. Therefore, a well-established striatal culture may be of significant value as a model for studying these and other conditions (Fig. 11). The major

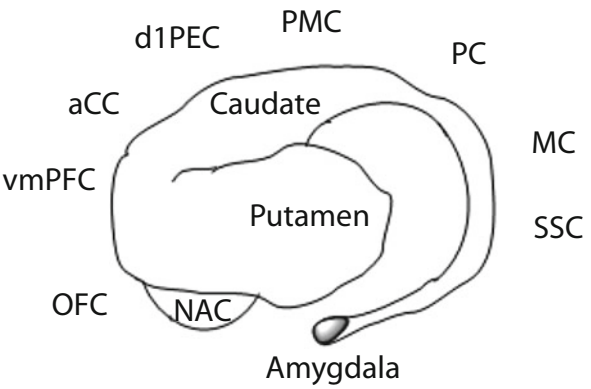

Fig. 11 Different compartments of the striatum. *NAc* nucleus accumbens, *vmPFC* ventromedial prefrontal cortex, *OFC* orbitofrontal cortex, *aCC* anterior cingulated cortex, *dlPFC* dorsolateral prefrontal cortex, *PMC* premotor cortex, *PC* parietal cortex, *MC* primary motor cortex, *SSC* somatosensory cortex

neuronal subtype in adult striatum is medium spiny projection neurons (MSNs), constituting ~95% of all striatal neurons and surviving via the inhibitory transmitter gamma-aminobutyric acid (GABA). They are characterized by medium-sized cell bodies, complex dendritic arbors, and a high density of dendritic spines that receive both glutamatergic and dopaminergic inputs. The remaining 5% of neurons are composed of GABAnergic aspiny interneurons.

6.1 Materials and Reagents

This material and reagents list is adapted from Naia and Rego (2018) with minor modifications:

- Round coverslips of 18 mm diameter, #1.5 (0.17 mm) thickness (Thermo Fisher Scientific, Menzel).
- Sterile non-tissue culture-treated Petri dishes of 35, 60, and 100 mm (VWR).
- 5, 10 ml serological sterile pipettes (Labbox).
- Sterile 0.2–10 µl, 10–200 µl and 100–1000 µl micropipette tips (Frilabo).
- 15 and 50 ml sterile conical centrifuge tubes (Corning).
- Sterile 1.5 ml microcentrifuge tubes (BIOplastics).
- Sterile syringes 20 ml (Terumo).
- Sterile 230 mm glass Pasteur pipettes (Labbox).
- Sterile 0.2 µm acetate cellulose filters (GE Healthcare, Whatman).
- Vacuum 0.2 µm filter bottle system, 500 ml (Corning).
- Cell strainers, 40 µm (Corning, Falcon®).
- Multiwells tissue culture-treated, flat bottom, polystyrene (Corning).
- Pregnant female mice (*Mus musculus*) with 16 days of gestation.

  NB: In this protocol, mice from FVB/NJ inbred strain (The Jackson Laboratory) were used.
- Hydrochloric acid (HCl), 37% (Sigma-Aldrich).
- Ethanol (absolute), 99.8% (Fisher Scientific).
- Isoflurane Iso-Vet 1000 mg g^{-1} Inhalation Vapour (Chanelle UK).
- Fetal bovine serum (FBS) (Thermo Fisher Scientific, GibcoTM).
- Trypan blue (TB) solution, 0.4% (Sigma-Aldrich).
- 5-FdU (Sigma-Aldrich).
- Anti-MAP2 (1:500; Santa Cruz Biotechnology).
- Anti-GABA (1:500; Sigma-Aldrich).
- Anti-DARPP32 (1:100; Abcam).
- Goat anti-rabbit IgG secondary antibody (Ab), Alexa Fluor 594 (1:200; Thermo Fisher Scientific, Invitrogen).
- Donkey anti-mouse IgG secondary Ab, Alexa Fluor 488 (1:200; Thermo Fisher Scientific, Invitrogen).
- Boric acid (H_3BO_3) (Sigma-Aldrich).
- Poly-D-lysine hydrobromide (Sigma-Aldrich).
- Potassium chloride (KCl) (Labbox).

- Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich).
- Sodium chloride (NaCl) (Merck).
- Sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich).
- Sodium phosphate monobasic (NaH_2PO_4) (Sigma-Aldrich).
- D-(+)-Glucose (Sigma-Aldrich).
- Sodium pyruvate (100 mM) (Thermo Fisher Scientific, Gibco™).
- HEPES (Sigma-Aldrich).
- Phenol red (Sigma-Aldrich).
- Deoxyribonuclease I (DNase I) (Sigma-Aldrich).
- Bovine serum albumin (BSA) (fatty acid-free) (Sigma-Aldrich).
- Trypsin, Type IV-S, from porcine pancreas (Sigma-Aldrich).
- Trypsin inhibitor, type II-S: Soybean (Sigma-Aldrich).
- L-Glutamine (200 mM) (Thermo Fisher Scientific, GibcoTM).
- Gentamicin (50 mg ml^{-1}) (Thermo Fisher Scientific).
- Neurobasal medium (Thermo Fisher Scientific, GibcoTM).
- B-27 supplement (50×), serum-free (Thermo Fisher Scientific, Gibco™).

6.2 Equipment

This equipment list is adapted from Naia and Rego (2018) with minor modifications.

- 11.5 cm fine straight scissor (Fine Science Tools, Dumont).
- 8.5 cm mini-dissecting scissors, straight with a sharp tip (World Precision Instruments).
- Medium forceps (Fine Science Tools, model: Dumont #7).
- Forceps with straight and angled fine tip (Fine Science Tools).
- Automatic pipettor and micropipettes.
- Glass Schott laboratory bottle with cap, 500 ml (DWK Life Sciences).
- Water bath with electronic temperature regulation.
- Ultrasonic cleaning bath (just for coverslip washing).
- Zeiss Stemi magnification glass (or equivalent).
- Phase contrast inverted microscope equipped with 10× and 20× objectives.
- Zeiss LSM 710 point-scanning confocal microscope (ZEISS, model: LSM 710 or equivalent).
- Hemocytometer (Sigma-Aldrich).
- Water jacketed CO_2 incubator.
- Vertical laminar flow chamber.
- Isoflurane vaporizer apparatus (E-Z Anesthesia, model: EZSA800 or equivalent).

6.3 Preparation of Buffers and Chemicals

This buffer and reagent preparation list is adapted from Naia and Rego (2018) with minor modifications.

Poly-D-Lysine for Coating (0.1 mg ml^{-1})
- Prepare a solution of 166 mM boric acid in type I (ultrapure, >17.4 mΩ resistance) water and adjust the pH to 8.2.
- Just before use, dilute the poly-D-lysine in the previously prepared boric acid solution and filter the solution in a laminar flow.

Hanks' Balanced Salt Solution
5.36 mM KCl
0.44 mM KH_2PO_4
137 mM NaCl
4.16 mM $NaHCO_3$
0.34 mM NaH_2PO_4
5 mM glucose
5.36 mM sodium pyruvate
5.36 mM HEPES
0.001% phenol red

Adjust the pH to 7.2 and filter the solution in a laminar flow using a vacuum filter system unit. Store at 4 °C, for no more than 1 month.

DNase I Solution (5 mg ml^{-1})
5 mg DNase I in 1 ml, 0.15 M NaCl.
Store at −20 °C in small aliquots for 1 week.
NB: Higher concentrations of this solution may result in a loss of enzymatic activity.

Bovine Serum Albumin Solution (3 mg ml^{-1})
150 mg BSA in 50 ml HBSS
In a laminar flow, filter the solution using a 0.20 μm acetate cellulose filter.
Prepare just before use.

Trypsin Solution (0.5 mg ml^{-1})
2.5 mg trypsin in 5 ml BSA solution.
Add 50 μl, 5 mg ml^{-1} DNase I solution.
In a laminar flow, filter the solution using a 0.20 μm acetate cellulose filter.
Prepare just before use.

Trypsin Inhibitor Solution (1 mg ml^{-1})

10 mg trypsin inhibitor in 10 ml BSA solution.
In a laminar flow, filter the solution with a 0.20 μm acetate cellulose filter.
Prepare just before use.

Washing Solution
Dilute the sterile trypsin inhibitor solution in BSA solution in 1:5 proportion to make a final volume of 10 ml.
Prepare just before use.

Inactivated Fetal Bovine Serum
To inactivate the FBS stock solution, warm it in a water bath at 56 °C for 30 min.
Aliquot the FBS and store at −20 °C until the shelf life expiration date.

Supplemented Neurobasal Medium
- Supplement the 500 ml bottle of Neurobasal medium with 1.25 ml, L-glutamine, and 0.5 ml gentamicin (a final concentration of 0.5 mM and 50 μg ml^{-1}, respectively).
- Make aliquots of 49 ml Neurobasal medium in sterile centrifuge tubes.
- Add 1 ml B-27 supplement (50×) to achieve a final volume of 50 ml. Once supplemented, the Neurobasal medium remains stable for up to 1 week if stored at 4 °C in a light-protected environment.
- On the day of culture, and only for the plating medium, add 0.25 ml inactivated FBS in 50 ml supplemented Neurobasal medium (0.5% final concentration).

6.4 Software

Fiji software (Image J, National Institute of Health, USA).

NB: Except for the coverslips, washing (described in the following Procedure (A), before poly-D-lysine coating) and brain dissection are done. All the procedures must be done in a sterile environment using a flow chamber.

6.5 Procedure

6.5.1 Coverslip Washing

1. Heat the coverslips in a loosely covered glass bottle embedded in 1 M HCl at 50–60 °C for 5–15 h with occasional swirling. Use a few coverslips at a time to forbid their sticking with each other. The acid washing protocol is recommended by the manufacturers for enhancing the cleanliness of the coverslip and increasing the cellular attachment.
2. Cool at room temperature (RT) and carefully decant the HCl solution into an appropriate container. Rinse thoroughly with ddH_2O before filling the glass bottle with ddH_2O. Sonicate in a water bath for 40 min. Perform the sonication twice.

3. Replace the ddH_2O with a solution of 50% ethanol (v/v) and sonicate in a water bath for 40 min. Perform the sonication step twice with an increasing concentration of ethanol: (i) 70% ethanol (v/v), (ii) 95% ethanol (v/v). Cover slips can be stored submerged in ethanol absolute ≥99.8% (v/v) for up to 6 months.
4. Before use, sterilize the coverslips by exposing them to UV light for ~15 min or using dry-heat sterilization.

6.5.2 The Protocol for Poly-D-Lysine Coating of Culture Vessels

- Thaw PDL in a 37 °C maintained water bath.
- Filter through a 0.22-micron filter.

Coat the plates with the following:

- 96-well plates, 50–60 μl $well^{-1}$.
- 4-well chamber slides, 300 μl $well^{-1}$.
- 24-well plates, 500 μl $well^{-1}$.
- 6-well plates, 2.5 ml $well^{-1}$.
- T25 flasks, 3–4 ml $flask^{-1}$.
- Incubate the coated plates at 37 °C for at least 2 h (best if left overnight).
- 1–2 h before the dissection, remove the Poly-D-Lysine via vacuum subjection and wash twice with sterile ddH_2O.
- The extra Poly-D-Lysine-coated plates could be stored at 4 °C for up to 1 month.

6.5.3 The Dissection of Brain and Striatum

This dissection protocol is adapted from Naia and Rego (2018) ***with minor modifications.***

- To minimize contamination, soak all the dissection tools in 70% ethanol (v/v) before commencing the dissection. Also, during the dissection, routinely dip the dissecting tools in 70% ethanol (v/v) for minimal contamination. Dry thoroughly before using.
- Anesthetize the pregnant female mice on the **16th gestational day** with **5% isoflurane (v/v)** and sacrifice it via cervical dislocation (or choose appropriate anesthesia as per the recommendations of the Institutional Animal Care and Use Committee). Place the mouse on its belly up and thoroughly rinse the abdomen with 70% ethanol (v/v). Collect the embryos by cesarean section by grasping the uterus with sterile forceps and cutting the connection using a mesometrium. Put the embryos in an HBSS-filled 100 mm Petri dish.
- Remove each embryo from the embryonic sac and rapidly behead it. Transfer the embryos' heads to an HBSS-filled 60 mm Petri dish before keeping it on ice.
- Using a pair of forceps, hold the embryo's head and insert the tip of a straight Dumont #5 forceps between the eyes to keep the head fixed above a surface. With the mini dissecting scissors pointed up, cut the skin and skull from the spinal cord terminal through the longitudinal fissure until the head cavity. Support the head in the Petri dish and gently squeeze the brain from the brain cavity in a frontal-

occipital direction using angled Dumont #5/45 forceps. Transfer one brain at once to a new HBSS-filled 60 mm Petri dish.

- Turn on the magnifying glass and adjust it for **~25× magnification**. With the brain faced upward, start the dissection by making a sagittal cut nearly 1 mm parallel to the midline using the angled Dumont #5/45 forceps. Use this cut to remove the meninges from the cortex. To stabilize the brain during dissection, use straight Dumont #5 forceps to gently hold the brain between the forebrain and the cerebellum.
- Open the slit using the same angled forceps to unfold the anterior cortex overlaying the lateral ventricle and the ganglionic eminences. The striatum, exhibiting an irrigated oval structure, is located in the lateral eminence. Use the tips of the angled forceps to cut out the striatum pinching in the vicinity. Transfer it to a sterile HBSS-filled 35 mm Petri dish.
- Repeat the procedures on the other lobe and for each brain till all strata are collected. The remaining tissue can be tied together to isolate the hippocampus, the cortex, or other brain regions of interest.
- For an easier understanding, Fig. 12 depicts a flowchart of the chronological sequence of tasks.

6.5.4 Dissociation and Plating of Striatal Cells

This dissection protocol is adapted from Naia and Rego (2018) with minor modifications.

1. Transfer the striatum into a sterile HBSS medium (7 ml) to a 15 ml Falcon tube.
2. Add 3 ml trypsin solution and mix by gently inverting the tube. Incubate for 2–3 min in a 37 °C water bath through a gentle and gradual mixing. Note that depending on the tissue amount and the trypsin activity, the trypsinization time may change. The current protocol is optimized for a 3 ml trypsin solution to handle six embryos; increasing embryos may require an adjustment instead of the total solution volume while maintaining the trypsin ratio.
3. Inactivate trypsin by adding a 5 ml trypsin inhibitor solution. Note that if the volume added in the previous step is changed, the volume of trypsin inhibitor solution should be added accordingly. Mix by gently inverting the tube and wait until tissue settles. Aspirate the medium.
4. Wash the cells by adding a 5 ml washing solution (trypsin inhibitor in BSA solution, 1:5) and mix by inverting the tube. Allow the tissue to settle and aspirate as much volume as possible without disturbing the pellet.
5. Add 2–3 ml supplemented Neurobasal medium with 0.5% FBS and gently homogenize with a P1000 pipette until the solution becomes homogeneous. Note that if the trypsinization step is adequate, aggressive trituration is not required, as too much physical stress on cells during mechanical homogenization is likely to reduce the cell viability.
6. Get ready a 1.5 ml Eppendorf with 20 µl cell suspension, 20 µl Neurobasal medium, and 20 µl TB solution, creating a threefold dilution factor. Use an inverted phase-contrast microscope to count the number of viable cells in a

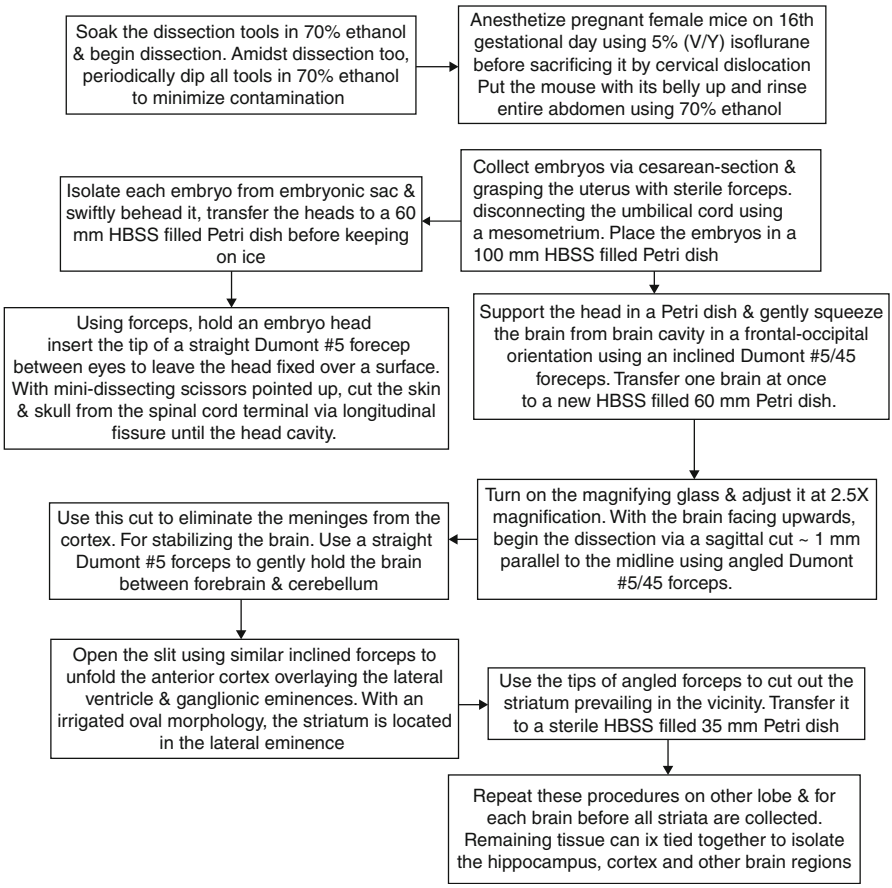

Fig. 12 Chronological sequence of steps involved in brain and striatum dissection

hemocytometer and plate at a density of 130 10^3 cells cm^2. For low-density cultures in coverslips, plate 92 $\times$ 10^3 cells cm^2. Neurons are maintained at 37 °C in a humidified incubator with 5% CO_2/95% air, for 12 days.

7. At DIV3, remove one-third culture medium and add an equal volume of freshly supplemented Neurobasal medium without FBS, containing 15 μM, 5-FdU (5 μM final concentrations) to reduce the dividing nonneuronal cells in primary culture. At DIV7, change the medium again by replacing half of it with a freshly supplemented Neurobasal medium devoid of FBS and 5-FdU. Feed cells every 3–4 days. Always do a 50% media change. Account for evaporation while keeping cells for long periods.

6.5.5 Characterization of Primary Striatal Cultured Cells

Characterize primary striatal culture in terms of neuron extent (MAP2 or NeuN-positive), glial cells (GFAP or S100 beta-positive), and GABAergic cells (GAD65 or

GABA-positive), MSNs (DARPP-32-positive and GAD65 or GABA-positive), and GABAergic interneurons (GAD65-positive and DARPP-32-negative).

6.6 Data Analysis

For striatal cultures characterization, six to eight images per individual experiment could be randomly taken using a confocal microscope for each labeled Ab. GABA- and DARPP-32-positive cells can be counted using Fiji software (Image J, National Institute of Health, USA) with Cell Counter plugin, and the percentage can be calculated relative to MAP2-positive cells.

7 Isolation and Primary Culture of Cells from Cerebellum

The cerebellum consists of two germinal layers. These are the ventricular zone (**VZ**) and the external germinal layer (**EGL**). While VZ consists of the **Purkinje cells** (major output neurons of the cerebellum), the EGL consists of the **granule cells**, the most abundant interneurons, that arise from the EGL. It must be noted that VZ is most active during embryonic development and prominently contributes to neurogenesis after birth.

However, the origin of other cells in the cerebellar cortex including **astrocytes, oligodendrocytes, stellate, basket, Lugaro**, and Golgi interneurons is not much clear. Based on the **tissue grafting** and **transplantation studies**, it is suggested that many cells that arise from VZ progenitors migrate into the cerebellar cortex after birth (Fig. 13). Nevertheless, whether each class of neuron and glial cell comes from a distinct progenitor or a common multipotent progenitor is still unknown.

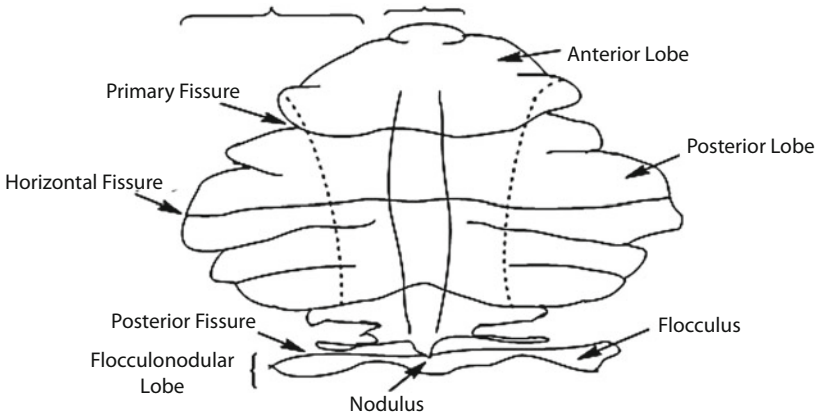

Fig. 13 The functional differentiated view of a cerebellum, controlling embryonic development through germinal layers and post-birth neurogenic actions

7.1 Culturing Usefulness of Cerebellar Neuronal Cells

There are several uses of cultured neuronal cells from the cerebellum. Briefly, the following points may be important. The culture of cerebellar granule neurons is relatively easier than the culture of Purkinje fiber.

7.1.1 Usefulness of Cerebellar Granular Neuron Culture

- The cell-intrinsic mechanisms underlying neuronal morphogenesis and connectivity have been recently studied using the cerebellar granule neurons. The role of key regulators of neuronal morphogenesis, such as **the transcription factors MEF2 and NeuroD, as well as the ubiquitin ligase Cdh1-APC and its target SnoN can be studied using granule neurons**.
- Towards an understanding of neuronal connectivity, granular neurons offer manifold benefits, such as the relatively simple cerebellar cortex architecture which may aid in translating molecular findings of individual cells into functional consequences, for a circuit.
- The culture of cerebellar granular neurons gives valuable information about **neuronal survival, migration**, and **differentiation**.
- The granular neuronal cells of the cerebellum are also utilized to understand the programmed cell death or **apoptosis** of neurons.
- Studies on the cerebellar neuronal cells have identified the key neuroprotective molecules including prosurvival protein AKT, growth factors like insulin-like growth factor 1 (IGF1), phosphatidyl-3-kinase, cyclic adenosine monophosphate, and myocyte enhancer factor 2.
- Based on the cell culture studies, it is now established that the key mediators of neuronal cell death are cyclin-dependent kinase 1 (CDK 1), c-Jun N-terminal kinase, and mammalian sterile 20-like kinase.
- These cell lines are also utilized to understand the basic electrophysiology of nerve signaling including depolarization, repolarization, hyperpolarization, and the basic electrophysiological roles of various ion channels, and neurotransmitters including those inhibitory neurotransmitters like **gamma-aminobutyric acid** (GABA).
- Granule neurons can also be used to understand **neuron cell migration**. It is observed that the granule neurons display a well-known maturation-dependent descent from the external to an internal granular layer of the cerebellar cortex. Fundamentally, these cells are of significant utility for uncovering glial guidance mechanisms sustaining neuronal migration. It is noted that neuron cell migration is governed by the adhesion molecules and members of the mPar6α polarity complex.

7.1.2 Usefulness of Cerebellar Purkinje Neuron Culture

Purkinje neurons are notoriously challenging to isolate and maintain in culture. However, recent improvements in culture techniques may increase their survival and differentiation in mixed cerebellar cultures, but their overall population in such cultures remains quite low. Purifying Purkinje neurons may require Percoll

sedimentation, which is more expensive and labor-intensive than the counterpart procedures in most neuronal culture protocols. Besides, the difficulties of culturing postsynaptic targets and postnatal cerebellar granules, neuron development complicates the study of granular neurons from the many knockout mice that die either at the embryonic stage or birth.

NB: The postnatal cerebellum is much easier to isolate than small regions of the embryonic cerebrum, and therefore, it is recommended that the postnatal development of granular neurons can be advantageous.

7.2 Process of Culture of Cerebellar Neuronal Cells

This protocol has been adapted from methods originally developed in the work of Messer (1977), Gallo et al. (1982), Thangnipon et al. (1983), D'Mello et al. (1997), and Dudek et al. (1997).

Herein, the following protocol describes the purification of a multipotent neural stem cell population from the postnatal cerebellum.

The protocol shows that this population can undergo self-renewal in culture and can generate neurons, astrocytes, and oligodendrocytes both in vitro and after transplantation. The findings suggest that cerebellar neurons and glia could be generated from a common progenitor. In addition, the approach used here may be applicable for isolating NSCs from other parts of the nervous system.

7.3 Materials and Reagents

The materials used in this protocol are adapted from methods originally developed in the work of Messer (1977), Gallo et al. (1982), Thangnipon et al. (1983), D'Mello et al. (1997), and Dudek et al. (1997).

- Animals: Rats, Long-Evans (postnatal day 6 [P6]) (one litter).
- HHGN dissection solution.
- AraC (Cytosine-1-β-D-arabinofuranoside) solution.
- 2 mg ml^{-1}DNase (0.5 ml aliquot for trituration).
- 1 M Glucose.
- Poly-L-ornithine-coated plates (of the desired size).
- 0.05% Trypsin.
- Basal Medium Eagle (BME).
- Culture medium for granule neurons.
- Trypan blue.

7.4 Equipment

The equipment used in this protocol has been adapted from methods originally developed in the work of Messer (1977), Gallo et al. (1982), Thangnipon et al. (1983), D'Mello et al. (1997), and Dudek et al. (1997).

- Surgical scissors.
- Fine forceps.
- Dissection microscope.
- Ice bucket and ice.
- Water bath preset to 37 °C.
- Falcon tubes.
- Pipettes.
- Centrifuge.
- Laminar flow hood.
- CO_2 incubator.
- CO_2 chamber.
- Hemacytometer.
- Micropipette with tips.
- Timer for timing incubations.

7.5 Method

This protocol has been adapted from methods originally developed in the work of Messer (1977), Gallo et al. (1982), Thangnipon et al. (1983), D'Mello et al. (1997), and Dudek et al. (1997).

7.6 Isolation of Cerebellum

- Prepare poly-L-ornithine-coated plates and solutions.
- Take Long-Evan rats (P6) and euthanize them using CO_2 chambers.
- In the next step, decapitate the pups.
- Decapitate the pups and isolate the cerebellum, begin by grasping a pup's head at the nose and inserting small surgical scissors into the foramen magnum (the aperture at the base of the skull through which the medulla and spinal cord extend).
- Proceed laterally, thereafter anteriorly through the ear, and curve towards the midline. On reaching the midline from one side, reinsert scissors into the foramen magnum and repeat the cutting procedure on the other side.
- The two incisions should meet in the midline.
- Using forceps carefully lift the skull upwards and flip it back until the hindbrain is exposed. Make sure that, as the skull is lifted, the brain remains at the base of the skull.

- If necessary, cut away any bone or other tissues that are obstructing the cerebellum peripheries. Extract the cerebellum with forceps and place it into a dish containing at least 5 ml HHGN dissection solution.
- In the isolation of the cerebella, use the dissection scope and fine forceps to remove the meninges as well as any large blood vessels or extraneous brain regions that are stuck to the cerebellum.
- *This step should be performed at a steady pace, with a plate of ice beneath the cerebellum plate. The meninges tend to come off quite easily if we start by pulling away residual choroid plexus from the ventral side, the typical site of the fourth ventricle.*

7.7 Dissociation and Culture of Cells from Cerebellum

- If one is working with two litters, an additional step of trituration is helpful.
- Working in the tissue culture hood, decant the cerebella dish into a 50 ml Falcon tube.
- Use a pipette to remove a major portion of the HHGN solution before adding at least 5 ml of fresh HHGN solution. Repeat this for a total of three washes.
- On removing the last HHGN wash, add 5 ml trypsin-DNase solution, ensuring a prior mixing of the solution through pipetting up and down. Place the tube in a 37 °C water bath for 10 min. Every 5 min, swirl the cerebella gently.
- Wash thrice with the HHGN solution.
- Prepare an aliquot of DNase for trituration by adding 250 μl of DNase to 5 ml of BME in a separate Falcon tube. Mix by pipetting.
- If one is working with two litters, one needs ~2 ml more of the DNase solution for additional trituration.
- Remove the last HHGN wash, and add the diluted DNase solution to the cerebellum. Dissociate by pipetting up and down with a 5 ml pipette as follows.
- For the first 15 rounds of pipetting, do not press the pipette tip against the very bottom of the tube. Instead, allow the cerebellum to fall apart into smaller chunks.
- Continue to pipette up and down, however, begin gently pressing the pipette tip against the tube bottom.
- On ~10 more pipetting rounds, the tissue pieces do not become any smaller. Press the pipette tip more firmly against the tube bottom and continue trituration until clumps of tissue go out of sight.
- To perform additional trituration, transfer the cell suspension to the ice for 5 min, allowing any residual undetected clumps to sink to the bottom. Transfer the top half of the cell solution to a separate Falcon tube and triturate the remaining again using a fresh DNase solution.
- Centrifuge cells at ~200 g for 5 min at 4 °C.
- Use a pipette to remove the supernatant. Add 5 ml culture medium for granule neurons.

- Pipette up and down until a homogeneous mixture is obtained. Add 10 ml more of culture medium incrementally, pipetting up and down on dispensing every 2–3 ml to ensure homogeneity.

  Place the cell suspension on ice and count a small aliquot using a hemacytometer:

  Make a 1:10 dilution of cells by adding 100 µl cell suspension to 650 µl culture medium and 250 µl TB.

  Count healthy, spherical cells with a halo. Do not count cells that take up the TB. *The expected yield is 10–15 million cells/cerebellum.*

  Plate the cells at the desired density. For morphological assays in 24-well plates with coverslips, use 17–20 million cells/plate. For biochemical experiments in 6-well plates, use 30 million cells/plate. Scale-up for larger plates. Maintain the cells by adding AraC solution ~24 h on finally plating to an extent of 10 µM. On the third day (in vitro), (DIV3), add 1 M glucose to make up 25 mM.

7.8 Isolation and Culture of Neuronal Stem Cells

Several diseases of the nervous system have been identified and well-characterized. These diseases may be responsible for the loss of sensation or motor functions, memory failure, or even loss of life. Traditional drugs may delay the complexity of these diseases, but are unable to regenerate the neuronal tissues or even restore the complete physiological functions of these neurons. Under these prevailing conditions, transplantation of the neuronal stem cells (**NSCs**) manifests as one of the important treatment modalities. It is further inevitable to understand the physiology and pathophysiology of the NSCs and their isolation and culture procedures, before knowing about the possible NSC application for regenerating the injured sites (Fig. 14).

As discussed in the following paragraphs, NSCs are available only in a certain restricted region of the nervous system. The stem cells, including NSCs, have the

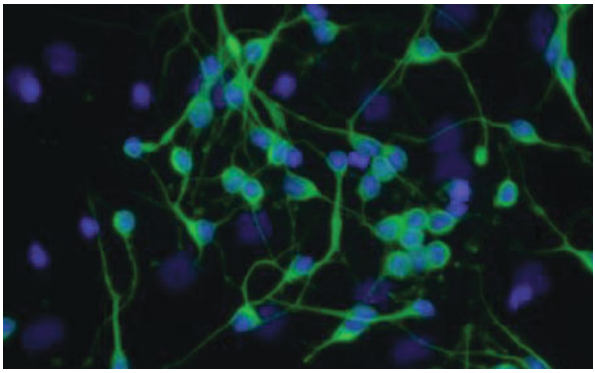

Fig. 14 Typical morphology of neural stem cells isolated from an adult mouse brain constituting the neurons. The blue stain indicates nuclei while the green one points out the cell-cell communication processes

capacity for both division (symmetric/asymmetric) and differentiation. In the case of symmetric division, one parental stem cell is equally divided into two new daughter stem cells. In asymmetric division, one parental stem cell divides into one new daughter cell and one specialized cell. The differentiation of NSCs gives rise to neurons, astrocytes, and oligodendrocytes. The NSC's niche or their microenvironment including exogenous cues influences them for their differentiation. Some neural cells are migrated from the subventricular zone (**SVZ**) along the rostral migratory stream which contains a marrow-like structure with **ependymal cells** and **astrocytes**. Both the astrocytes and the ependymal cells arise from glial tubes and are used by migrating neuroblasts.

The following paragraphs describe the location of NSCs in the brain, the types of NSCs, and their origin.

7.9 Location of Neuronal Stem Cells in the Brain

NSCs are present only in the restricted regions of the vertebrate brain. These cells continue to divide throughout the life span of a vertebrate.

The NSCs are predominantly located in the following regions of the mammalian brain.

The Subgranular Zone in the Hippocampal Dentate Gyrus
The Subventricular Zone Around the Lateral Ventricles
The Hypothalamus (precisely in the dorsal α1, α2 region, and the "hypothalamic proliferative region").

7.10 Types of Neuronal Stem Cells and Their Origin

The NSCs are broadly divided into **embryonic stem cells** and **adult stem cells.** Here is a brief discussion about them.

7.10.1 Embryonic Stem Cells

The embryonic stem cells (ESCs) are the pluripotent cells that have the capability of differentiating into any cell type.

7.10.2 Adult Stem Cells

As mentioned in the above paragraphs, the NSCs are located in certain brain regions of an adult person. Adult NSCs differentiate into new neurons within the adult SVZ, a remnant of the embryonic germinal neuroepithelium as well as the dentate gyrus of the hippocampus. Adult stem cells are limited in their ability to differentiate, with a tendency to exhibit a decrease in number and proliferation ability over time, as a result of aging. Various approaches are known and practiced to counteract this age-related decline. As FOX proteins regulate neural stem cell homeostasis, they are used to protect neural stem cells by inhibiting Wnt signaling.

7.11 Important Initial Discoveries Related to the Culture of Neuronal Stem Cells

- **In 1965**, Altman and Das used the [3H]-thymidine incorporation assay for the first time to ascertain the involvement of a certain region (postnatal hippocampus) of the young adult rat brain in neurogenesis (Altman and Das 1965).
- **In 1989**, *Temple and colleagues* analyzed the subventricular zone of the mouse brain and showed the presence of multipotent, self-renewing progenitor stem cells (Temple 1989).
- **In 1992**, *Reynolds* and *Weiss* analyzed adult rat brain tissues (striatal tissue, including the SVZ) and showed for the first time that in in vitro conditions the epidermal growth factor (EGF) induced the NSC proliferation (Reynolds and Weiss 1992).
- Two years later, the same group noticed that in the in vivo conditions the subependymal region in the mouse brain is the source of NSCs. Based on the earlier results, Weiss further reported that EGF and basic fibroblast growth factor (bFGF) cooperatively induce NSCs proliferation, self-renewal, and expansion. These experimental NSCs were isolated from an adult mouse thoracic spinal cord.
- **In 1992**, *Cepko* and *Snyder* were the first to isolate multipotent cells from the mouse cerebellum alongside illustrating their stable transfection with the oncogene v-Myc (Snyder et al. 1992).
- The v-Myc is one of the genes that are now widely used to reprogramme the adult non-stem cells into pluripotent stem cells.
- NSCs can grow in single-cell suspensions retrieved via enzymatic digestion and form spherical clusters called **neurospheres**, which are nonadherent and can be replated in a selective culture medium to obtain neural cells. Neurospheres can also be subcultured to expand the NSCs pool for manifold experimental or therapeutic purposes. Periventricular regions and olfactory bulbs in adult mammalian brains are rich NSCs sources.
- NSCs can also be isolated and separated via FACS analysis (cell sorting).

7.12 In Vitro Source(s) of Neuronal Stem Cells

NSCs can be derived from the following different sources using the latest technical modifications.

- Direct isolation and culture from the neuronal tissues
- Differentiation of pluripotent stem cells to neuronal stem cells
- Transdifferentiation of somatic cells to neuronal stem cells

Overleaf is a brief discussion about the isolation and culture of neuronal stem cells.

7.12.1 Direct Isolation and Culture from the Neuronal Tissues

As mentioned earlier, certain regions of the embryonic as well as adult mammalian brain such as the subventricular zone (SVZ) contain NSCs and exhibit a capacity to proliferate and differentiate. Additionally, most of the cortical interneurons and particularly GABAergic interneurons of the developing brain originate from the ganglionic eminence. However, the use of ESCs or iPSCs (induced pluripotent stem cells) to generate NSCs is less invasive than the use of the animal brain. The use of these cells has some limitations too. For example, ***the protocols for inducing the ESCs and iPSCs differentiation towards neuronal phenotypes are always time- and cost-consuming***. Additionally, the success rate of ESCs or iPSCs use is 70–80% Direct isolation of NSCs from the animal brain is 99% nestin-positive. The most disappointing factor is that the cultured stem cells lose their genetic stability and differentiation ability on several passages. Contrary to these shortfalls, research suggested the conversion of somatic cells to NSCs, although these specially generated cells are off-limits for every stem cell research laboratory due to a lack of resources. Thus, surgical isolation of NSCs directly from the animal brain is still in demand, and with improved surgical techniques, isolation of more undamaged viable cells is presently feasible.

7.12.2 Process of Primary Neural Stem Cell Culture

This protocol was adapted from the method by Polleux and Ghosh (2002***).***

The most important positive point of this technique has provided the means to monitor physical characteristics. Additionally, this technique can manipulate the genetic and epigenetic factors that regulate the capacity to self-renew and differentiate into defined cells of the nervous system. The defined cells here comprise neurons, astrocytes, and oligodendrocytes. In the presence of **EGF** or **bFGF**, these cell populations can be reliably expanded and maintained as neurospheres, and once these growth factors are removed, the three major CNS cells are efficiently generated.

- Based on the 1992 *Reynolds and Weiss's* estimation, the total percentage of neural stem cells is quite small (<0.1% in adults). Thus, it is often important to maximize the yield of these cells from the animals. As indicated in the previous paragraphs, it is generally recommended that trypsin may be toxic to the neuronal cells and therefore papain may be used as an alternative due to its low toxicity. The age and plating density of the cells is also important for successful culture.
- At present, ***the generation of mouse strains expressing fluorescent proteins under the control of neural progenitor specific markers, Nestin-EGFP, Sox2-EGFP and hGFAP-EGFP, coupled with an ability to efficiently isolate the cells using FACS, has enabled the culture of highly enriched neural progenitor cell populations and using the NSA, the evaluation of the proliferative, self-renewal, and multipotential capacities of these cells*** **in vitro** ***has also become feasible***.
- *Reynolds and Weiss* isolated and cultured the NSCs from an adult brain and use the **neurosphere assay (NSA)**. Due to the serum-free composition of the cell

culture medium, the majority of the harvested cells from the periventricular region die within 3 culture days. According to Reynold and Weiss, under these serum-free conditions, <0.1% of cells survive and respond to EGF, alongside undergoing an active cell proliferation phase (Reynolds and Weiss 1992).

- In Reynolds and Weiss's experiments, the adult NSCs cultured in a serum-free medium in presence of EGF, proliferated to form a ball of undifferentiated cells called a **neurosphere**. However, these undifferentiated cells could differentiate to form more numerous secondary spheres. These cells can also be induced to differentiate, generating the three major cells of the CNS. Their experimental results offered substantial validation that the NSCs isolated from the adult brain has the capacity for proliferation, self-renewal, and the ability to give rise to several differentiated, functional progeny.
- Besides growth factors such as EGF and bFGF, other factors that affect the proliferation of NSCs include antioxidants, nutrients, and lipids. For the optimized growth of NSCs, a specialized serum-free medium was developed which is named **STEMPRO NSC SFM**.
- Thus, it is now confirmed that it is possible to produce a consistent and renewable source of undifferentiated CNS precursors (a portion of which are stem cells) by using specialized medium and growth factors. These precursors could be well differentiated into neurons, astrocytes, and oligodendrocytes (Gritti et al. 1996; Reynolds and Weiss 1996; Weiss et al. 1996).

A detailed protocol for isolation and culture of NSCs is described by O'Connor et al. (1998), Rietze and Reynolds (2006), Shin and Vemuri (2009), Guo et al. (2012), and Louis et al. (2013). Readers are suggested to go through these publications for detailed knowledge of the respective protocols.

The following research protocol is adapted from the method by Polleux and Ghosh (2002) with modifications.

7.12.3 Materials and Reagents

The materials and reagents list is adapted from the method by Polleux and Ghosh (2002).

- Dulbecco's phosphate-buffered saline (PBS), 1× sterile (Sigma).
- Enzyme solution for neurosphere assay (pre-warmed).
- Heavy inhibitory solution (HI) (pre-warmed).
- Laminin stock solution (1 mg ml^{-1}) (Sigma).
- Store stock solution in 0.1 ml aliquots at −20 °C. Just before use, prepare a 1:50 dilution by diluting 0.1 ml stock solution in 5 ml sterile 1× PBS supplemented with Ca^{+2} and Mg^{+2} (Sigma).
- Light inhibitory solution (LI) (pre-warmed).
- Mice (embryonic or adult).
- NEP basal medium.
- NEP basal medium containing 2% horse serum (heat-inactivated for 30 min at 60 °C, Invitrogen).

- NEP complete medium.
- Neurobasal medium (PSG).
- Paraformaldehyde (PFA; Sigma), 4% in 1× PBS.
- Poly-D-lysine (Sigma).
- Reconstitute in H_2O for a stock solution of 1 mg ml^{-1} and store in 1.0 ml aliquots.
- Trypsin-EDTA (Sigma) (optional).

7.12.4 Equipment

- Centrifuge.
- Dishes, polystyrene (6 or 10 cm non-treated) (BD Falcon).
- Dissection tools for removing mouse brain from the skull.
- Forceps, sterilized (five in number, pointed) (Fine Science Tools 11252-30).
- Hemocytometer.
- Incubator preset to 37 °C (humidified, 5% CO_2).
- Knife, microsurgical (5 mm) (MSP/Surgical Specialties 7516).
- Parafilm.
- Pipette (P200; P20 may be used in place of P200).
- Plates (polystyrene, 96-well flat bottom), low cell binding (Corning 3474).
- Sterile razor blade.
- Scissors, sterilized micro-spring (8.5 cm) (Fine Science Tools 15,009–08).
- Slides, eight-well chamber (Nunc 177402).
- Transfer pipettes, sterile disposable (Fisher).
- Tubes, sterile conical (15 and 50 ml).
- Tubes, sterile microcentrifuge (1.5 ml).
- Water bath preset to 37 °C.

7.12.5 Methods of Primary Neural Stem Cell Culture

The method of this protocol is adapted from the method by Polleux and Ghosh (2002).

Dissection

- Carefully remove the brain from the skull of an embryonic or adult mouse and place it in a clean dish containing ice-cold 1× PBS.
- To dissect the tissue from an early embryo (**<E16**), separate the two brain hemispheres. Carefully separate the region of interest (e.g., dorsal telencephalon) using a microsurgical knife.
- Carefully isolate the meninges from the tissue using fine-tipped forceps and a microsurgical knife. Meninges must be removed from the tissue since they will not digest efficiently in an enzyme solution.
- To collect tissue from a late-stage embryo (**>E16**) or adult using a razor blade, cut a coronal brain slice containing the region of interest (lateral ventricle, hippocampus, etc.).
- Using a sterile transfer pipette, carefully transfer the tissue to a non-treated polystyrene dish containing 1× PBS in a cold state. To speed up enzymatic digestion, cut the tissue into smaller pieces using the micro-spring scissors.

- Using a sterile transfer pipette, transfer the tissue to a 15 ml conical tube containing a 10 ml enzyme solution. Minimize the amount of transferred PBS with the sample. Incubate at 37 °C for 20 min, mixing diligently every ~5 min.
- Do not vortex.
- Add another 10 ml enzyme solution. Incubate for 20 min at 37 °C, mixing occasionally.
- Incubation times may vary. The tissue is ready on achieving a thick and viscous consistency.
- In a sterile hood, carefully remove the enzyme solution using a pipette, leaving the tissue at the bottom of the tube.
- Add 4.5 ml Light Inhibitory (LI) solution to the tube. Carefully flick the tube, remove the solution, and repeat with another 4.5 ml LI solution.

  Caution: The tissue will slowly dissolve well and should not be mixed with a pipette.
- Remove the LI solution, leaving the tissue at the bottom of the tube, and add 6 ml Heavy Inhibitory (HI) solution. Incubate for 2 min at 37 °C. Gently remove the HI solution.
- Add 5 ml NEP basal medium, flick the tube, and remove the medium.
- Add 0.5–1.0 ml NEP complete medium and triturate 10–20 times, until the tissue pieces are dissociated.
- More medium may be needed, depending on the amount of tissue used.
- Count the cells using a hemocytometer and add an appropriate number to a non-treated polystyrene dish containing NEP-enriched medium.
- Cell density should be 1 10^6 cells/6-cm plate or 2 × 10^6 cells/10-cm plate. However, the plating density varies as per the age points and brain regions.
- Incubate cells in a humidified 37 °C incubator (+5% CO_2). Monitor the dishes daily for neurosphere formation.
- Adult lateral ventricle neurospheres take ~1 week to form, while embryonic neurospheres are observed in a few days.
- Once spheres have formed, replace the medium every 3 days by transferring the spheres to a 15 ml conical tube and letting them settle via gravity at 37 °C (centrifugation is not recommended). On getting settled, remove the medium and replace it with a fresh NEP complete medium. Transfer spheres to a fresh dish.

Slide Coating

- Just before use, dilute the poly-D-lysine stock solution at 1:50 in H_2O. Add 0.2 ml diluted poly-D-lysine to each well of an eight-well chamber slide. Incubate for 1 h at RT.
- Wash wells thrice with H_2O. Add 0.2 ml diluted laminin to each well and incubate overnight at 4 °C.
- Store slides at 4 °C until use and subsequent wrapping in parafilm to minimize evaporation.

- Before use, remove laminin and wash wells once with Neurobasal medium (PSG).
- Do not remove laminin from the wells until immediately before use.

Differentiation

- Add 100 μl NEP complete medium to each well of a poly-D-lysine/laminin-coated eight-well chamber slide. Transfer one sphere to each well using a P200 pipette and incubate overnight at 37 °C.
- On 24 h, ensure spheres have attached to the slide and carefully remove the medium. Add 200 μl NEP basal medium containing 2% heat-inactivated horse serum.
- Culture for 2–3 days, changing the medium on daily basis.
- For fixation and subsequent immunohistochemical analysis, remove the medium. Rinse once with 1× PBS before fixing with 4% PFA in 1× PBS for 1 h at 4 °C.

Secondary Neurosphere

- Using a P20 or P200 pipette, mechanically dissociate a single neurosphere in a 1.5 ml microcentrifuge tube.
- If this is difficult, incubate spheres in trypsin for 5 min and dissociate mechanically.
- Centrifuge the cells at 3000 rpm for 5 min and resuspend in NEP complete medium.
- Plate the cells individually in 96-well, low cell-binding plates or bulk passage multiple spheres.

NB: Human NSCs double every 48–72 h. Rodent NSCs double every 24 h. Cells should be split from 1:2 to 1:6 once being 80% confluent, roughly every 3–5 days.

In general, NSCs loosely adhere to the culture medium, and it is not uncommon to have cells and cell debris floating in the culture media. As long as the adherent cells on the plate are growing homogeneously in a monolayer regime, there is no risk of the floating cells as these are likely to be washed away during media changes.

NSCs can be cultured as adherent cells, wherein they produce large clones containing neurons, glia, and additional stem cells. However, they can also be cultured as floating, multicellular neurospheres. As described earlier, the isolation of NSCs from the CNS through the neurosphere formation assay (NFA) was first demonstrated in 1992 (Reynolds and Weiss 1992). Since the 3D structure of the neurosphere creates a niche that is more physiologically relevant than 2D culture systems, the neurospheres gradually became an assay of choice and a readily accepted scheme for isolating and propagating NSC.

A low yield of NSC is often a result of (1) poor adhesion using the wrong version of PBS (Ca and Mg devoid); (2) improper storage of STEMPRO NSC SFM complete medium (repeated warming and cooling compromises the growth factors); (3) prolonged enzyme treatment diminishes the viability. Make sure to use the right buffer solution for Cell Start and make an aliquot of the complete medium for the

day's warming. Expose NSC to enzyme solution just for initiating the disaggregation.

Unlike most cell culture systems, the NSCs mandate a denser plating and concomitantly emerge as highly proliferative. Proliferation is reduced in low-density cultures presumably due to a loss of cell-to-cell contact. Passaging the cells at a 1:2 proportion is ideal; however, if the cells are growing nicely, it is possible to split them at 1:3. Still, the clustering of cells sometimes happens due to (1) the effect on coating if the dish dries out; (2) a too high plating density; and (3) improper cell dissociation at the time of passage.

Also, the detachment of cells sometimes happens as a result of (1) sudden temperature change of the medium; (2) fixative (4% PFA) going bad; and (3) bacterial contamination. If cells are loosely attached to the substrate, apply the two-step fixation (the standardized protocol). If open handling (nonsterile environment) is desired, add the preservative to the staining solution.

While growing under the specific culture conditions and in presence of EGF and/or bFGF, NSCs and progenitor cells grow and expand in an undifferentiated state and form neurospheres. Cells inside the sphere rarely exhibit a differentiated morphology or antigenic properties, typical of neuronal or glial cells. The vast majority of cells are immunoreactive for nestin, an intermediated filament characteristic of undifferentiated neuroepithelial cells. However, on the removal of growth factors and plating stem cell progeny onto a good adhesion substrate (such as poly-D-lysine, laminin, or Matrigel), neurosphere-derived cells differentiated into neurons, astrocytes, and oligodendrocytes. The procedure described below is a basic one, as per which, the cells are differentiated in a serum-free medium, following the same protocol as used to establish enriched neuronal cultures from the embryonic rodent brain. Overall, two methods are described for neurosphere differentiation: whole spheres cultured at low density (typically demonstrating the multipotent individual spheres) or as dissociated cells at high density (typically determining the relative percentage of differentiated cells). This approach provides the culture conditions to test the effect of specific molecules on the differentiation of stem cells along with their neuronal and glial progeny.

7.12.6 Differentiation of Pluripotent Stem Cells into Neuronal Stem Cells

Neural induction is the process in which the human pluripotent stem cells (hPSCs) including ESCs and iPSCs are differentiated into neural stem cells (NSCs). These cells may have the capacity of pluripotency. The neural induction of human ESCs and iPSCs is characterized by the coculture with a stromal cell line or embryoid body (EB). However, some recent studies observed that neuronal induction of ESCs and iPSCs can also be possible in monolayer culture in a defined matrix before exposure to inductive factors. Pluripotent stem cells, including ESCs and iPSCs, can generate the desired cells through differentiation and forms significant alternatives to isolated primary cells. In general, the protocols for neural differentiation from PSCs can be categorized via embryoid body (EB) formation and monolayer culture (Hu et al. 2010).

Embryoid Body Formation

For the formation of the embryoid body, both the suspension and adherent culture can be used. Initially, the detached ESC and iPSC colonies are grown in suspension culture. Later on, the EBs are plated on adhesive substrates in a defined serum-free medium that promotes the generation of neural tube-like rosettes along with the selection of neural progenitor cells (**NPCs**).

NB: For the generation of NPCs from hPSCs, the Neural Induction Medium (STEM diff™) is utilized. It is a defined serum-free environment for the generation of NPCs from hPSCs. This medium is used for EB formation and selection of neural rosettes, to obtain highly enriched cultures of CNS-kind NPCs within ~12 days.

Monolayer Culture

In contrast to EB culture, NSCs can also be cultured as a monolayer. In this case, the PSCs directly differentiate into NSCs via the neuronal rosette stage. To promote differentiation, the serum-free medium is used as a nutrient-deficient environment with specialized growth factors or inhibitors. A comparative morphology, as well as marker protein expression between EB culture and monolayer culture, revealed no significant mutual distinctions.

7.12.7 Transdifferentiation of Somatic Cells into Neuronal Stem Cells

In the year 1974, *Selman and Kafatos* for the first time used the term **transdifferentiation.** In this event, **the lineage-specific transcription factor** and **chemical compounds** are used to transform one kind of mature somatic cell into another mature somatic cell without any need of passing through an intermediate pluripotent state.

7.12.8 The Following Factors Affect the Transdifferentiation of Various Cells to Neuronal Stem Cells

Tissue factor-induced transdifferentiation
Chemical compound-induced transdifferentiation
Growth factor-induced transdifferentiation
Three-dimensional culture-induced transdifferentiation

Ahead is a brief discussion about these factors.

Tissue Factor-Induced Transdifferentiation

Ding and others for the first time experimentally showed that tissue/pluripotency factors in combination with other optimum signaling inputs induced the NSCs formation from the mouse fibroblasts. Interestingly, it was observed that a single tissue factor such as *Sox2* or *ZFP521* can generate iNSCs from mouse and human **fibroblasts.** Some other cells that are utilized to form iNSCs are **astrocytes, adult liver cells, cells from cord blood, B lymphocytes, Sertoli cells, and urine epithelial-like cells**. Based on these findings, new strategies can be developed for generating the iNSCs through direct cell transdifferentiation following **virus-mediated exogenous gene expression**.

Chemical Compound-Induced Transdifferentiation

Various chemical compounds are screened to manipulate and reprogramme cells' fate, resulting in claims that the chemical compounds may have various added advantages contrary to exogenous virus-induced transdifferentiation. These experiments also provided evidence that to generate NSCs, it is not necessary to provide exogenous virus genes. One of the best examples of this is the use of a cocktail of **Repsox CHIR99021** and **valproic acid (VPA)**. This mixture inhibits and transforms growth factor (TGF)-β, histone deacetylases (HDACs), and glycogen synthase kinase (GSK)-3. Under hypoxic conditions, *this mixture* generates the NPCs (ciNPCs) from mouse embryonic fibroblasts. The same chemical mixture is also successfully used to generate ciNPCs from mouse tail-tip fibroblasts and human urinary cells. Experimental evidence also demonstrated that in absence of hypoxic conditions, eight distinct chemical mixtures transdifferentiated the mouse fibroblasts into NSC-like cells. The chemical mixture is as follows: **Pamate** (a histone demethylase inhibitor), **LDN193189** (an inhibitor of the BMP type I receptor ALK2/3), **CHIR99021, Hh-Ag1.5** (a potent smoothened agonist), **A83-01** (an inhibitor of the TGF-β type I receptor ALK4/5/7), **RG108** (a DNA methyltransferase inhibitor), and **RA, SMER28** (an autophagy modulator). In another study, a cocktail of **Repsox, SB431542** (a selective TGF-β receptor I inhibitor, such as ALK4 and ALK7), **Dorsomorphin** (a selective BMP signaling inhibitor), **Forskolin** (an adenylyl cyclase activator), **Parnate**, and **VPA** is utilized to induce neural crest-like precursors from mouse embryonic fibroblasts. Another chemical mixture that generated the ciNSCs consists of **Thiazovivin** (a selective Rho-associated protein kinase inhibitor), **VPA, A83-01**, and **Purmorphamine** (a smoothened receptor agonist). It is observed that while some chemicals are commonly used by various researchers, newer and newer chemicals are also being used for the first time. The common targets of HDACs, GSK-3, and TGF-β are widely being used, in recent times. The mechanism of action of these three signaling molecules and their importance are well established. For example, while GSK inhibitors regulate Wnt signaling, the TGF-β inhibitors control the mesenchymal to epithelial transition (EMT), and the HDAC inhibitors cause chromatin de-condensation and induce cells into a plastic state. However, the mechanism underlying chemical-induced transdifferentiation remains majorly unknown.

Growth Factor-Induced Transdifferentiation

Various growth factors that predominantly act as mitogens are utilized to induce transdifferentiation. Some of the most prominent growth factors utilized for this purpose are the epidermal growth factor (**EGF**) and fibroblast growth factor (**FGF**) and **bFGF**. Of note, besides growth factors, other factors are also required for transdifferentiation. Careful observation showed that during the direct induction process, cells first pass through a transient and partially reprogrammed state which is followed by the cell transdifferentiation through a safe, nonintegrated, and efficient method.

Three Dimensional Culture-Induced Transdifferentiation

In in vivo conditions, stem cells need a special niche to grow. On the other hand, two-dimensional culture conditions fall short to provide this specialized environment. Therefore, a three-dimensional in vitro cell culture system should mimic the complex physical environment, enhancing the NSC self-renewal and multipotency. Using nonadherent substrates, the mouse fibroblasts can be converted into three-dimensional spheres. This sphere exhibits the traits of neural progenitor-like cells in terms of morphology, self-renewal ability, and specific marker expression (e.g., enhanced level of Sox2 expression). The three-dimensional scaffolds (such as graphene foam, a porous and biocompatible scaffold) might also be used for iNSC generation.

8 Characterization of Neuronal Stem Cells

Various markers including positive and negative phenotypic signatures are identified to characterize NSCs. However, the NSCs can differentiate into neurons, oligodendrocytes, and astrocytes. Many of these markers are related to the stem and progenitor state with the absence of a still greater differentiated phenotype. While many of the marker proteins are predominantly expressed by neuronal stem cells, some marker proteins are common to nonneuronal cells. For instance, the **CD133** (used to sort precursor cells), **Ki67** (general proliferative marker), and **Nestin** (class IV neurofilament) are highly expressed in neuronal cells. The NSCs also express **Sox1** and **Sox2** are HMG box and Msi1 (Musashi 1, a noted marker) that are not expressed by the differentiated cells. Of note, **GalC, NG2, O4, myelin basic protein, CNPase,** and **RIP** are expressed in oligodendrocyte progenitors or oligodendrocytes. The **neurofilament, NCAM, MAP2, β III tubulin, NeuN, HuC/D,** and **Dcx** are expressed in neuronal progenitors or fully differentiated neurons. The proteins **CD44, GFAP,** and **S-100** are expressed in astrocyte progenitors or fully differentiated astrocytes.

8.1 Flow Cytometry Analysis of Isolated and Cultured Neuronal Stem Cells, Mature Neuronal Cells, and Glial Cells

NOTE: The expression of specific NSCs markers in neurons and glial cells can be assessed using a flow cytometry assay.

The following protocol is based on a publication by Menon* et al *with minor modifications (Menon et al.* 2014*).

- Dissociate neurospheres or detach cultured NSCs from coated plates by trypsinization to isolate them as single cells. Add 500 μl fixation buffer (2% paraformaldehyde (PFA) in PBS) to 100 μl cell suspension (around one million cells are enough for staining with each Ab) and then incubate the tubes at RT for 15 min.

- Wash the cells by adding 1 ml PBS to the tube and centrifuge at 110× g for 5 min. Discard the supernatant and resuspend the cell pellet, leaving approximately 100 μl in the tube.
- Permeabilize the cells with Tween-20 (0.7% Tween-20 in PBS) by adding 500 μl Tween-20 buffer to 100 μl cell suspension. Incubate the tubes at RT for 15 min on an orbital shaker (100 rpm).
- Repeat the centrifugation and the PBS wash. Remove the supernatant from the tubes completely, leaving only the pellet behind. Add 100 μl diluted primary Abs (antibodies) (rabbit anti-mouse βtub-III, GFAP, and Nestin antibodies at 1:200 concentration) in dilution buffer containing 1% BSA, 10% serum (goat serum), and 0.5% Tween-20 in PBS and gently triturate to mix. Incubate the tubes at RT for 30 min on an orbital shaker.
- Wash the cells once with PBS and remove the supernatant from the tubes completely, leaving only the pellet behind.
- Add 100 μl diluted secondary Ab (goat anti-rabbit FITC conjugated at 1:500 concentration) in PBS to the cell pellet and gently triturate to mix. Incubate the tubes at RT for 30 min on a shaker in the dark.
- Wash the samples 2× with PBS and then wash once with flow buffer. Centrifuge and resuspend the cells in ~150 μl flow buffer for flow cytometer analysis.

8.2 Immunolabeling Distinguished Neuronal Cells, Neuronal Stem Cells, and Glial Cells

The identification protocol was observed by Menon et al. (2014).

- Fix the cells with 4% paraformaldehyde (in PBS, pH 7.2) for 20 min at RT (0.5 ml well^{-1} for 24 well and 0.1 ml well^{-1} for 96 wells).
- Remove the paraformaldehyde solution using an aspiration system connected to a vacuum pump.
- Add PBS (pH 7.2) to the cells and incubate for 5 min.
- Aspirate PBS using a vacuum pump and repeat this washing two more times for a total of three wash steps.
- Saturate nonspecific locations and permeabilize with PBS containing 0.1% Triton-x-100 and 10% normal goat serum (NGS) for 60 min at RT.
- Incubate the cells with primary Abs: **For neuron, microtubule-associated protein-2 (MAP-2), neuron, β-tubulin type III, and doublecortin; for astrocyte, glial fibrillary acidic protein (GFAP, S100-β); and for oligodendrocyte, myelin basic protein (MBP, O4, and GalC) diluted in blocking solution for 1 h at RT (or overnight at 4 °C).**
- Wash cells thrice with PBS.
- Incubate the cells with the secondary Abs (Molecular Probes, Life Technologies, 1:1000) diluted in blocking buffer for 60 min at RT.
- Wash the cells thrice with PBS; include DAPI (1:1000) in the second wash for a nuclear counter stain.

- Mount on the slides using Fluoromount fluorescent mounting media (Sigma, Cal. No. F4680).
- Visualize the immunostained cells/cell proteins under a fluorescent microscope using appropriate fluorophore filters.

9 Isolation and Culture of Glia or Supporting Cells

9.1 Culture of Microglial Cells

Microglial cells originated from the myeloid progenitor cells of the embryonic yolk sac and maintain their population throughout the life span of an individual via self-renewal. So, the microglia population in the brain has no direct relationship with the peripheral blood monocytes/macrophages. ***Much pioneering research on microglia functions has been carried out* in vivo *using genetically engineered animal models***.

However, ***to fully understand the role of microglia in neurological and psychiatric disorders, it is crucial to study primary human microglia from brain donors***.

The Microglia are key players in the CNS, both in healthy and in diseased states. This brain-residing phagocytic cells perform multiple roles in CNS homeostasis including the events like neurodevelopment, synaptic plasticity, damage, inflammation, or infection within and outside the CNS and, therefore, brain immunity. In course of normal aging, the microglial phenotype shifts to an active-prone state, a characteristic of neurodegenerative disorders such as **Parkinson's disease (PD), Alzheimer's disease (AD)**, and **multiple sclerosis (MS)**. Therefore, microglial cells exhibit both beneficial and adverse effects on CNS. Various genetically engineered mouse models screened via in vivo experiments are used to examine the role of microglia cells in health and diseases. For ex vivo experiments, immunohistochemistry is a widely used analysis module. However, while animal model experiments do display certain similarities with human settings, considerable distinctions are manifested in the different human CNS disorders. This is substantial because of some exclusive challenges encountered in creating all human CNS diseases within the animal models. Therefore, the primary culture of microglial cells from the human brain is essential to understanding human physiology and pathology, from the perspective of these cells. Generally, the postmortem grade human brain is used to isolate microglia cells and astrocytes via enzymatic digestion, immune pairing, or magnetic sorting (Jana et al. 2007).

9.2 Protocol for Isolation and Culture of Microglial Cells

The materials and reagents described here have been adapted from Lian et al. (2016).

9.3 Materials and Reagents

- 15 ml centrifuge tubes (Corning).
- 50 ml centrifuge tubes (Corning).
- 12-well plates (Corning, Costar®).
- Mouse pups (newborn) (P0-P2).
- PDL (Poly-D-lysine) (Sigma-Aldrich).
- Ethanol.
- DMEM (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific).
- Fetal bovine serum (FBS) (GE Healthcare, Hyclone™).
- Penicillin-streptomycin (PEN/STREP) (10,000 U ml^{-1}) Thermo Fisher Scientific).
- Hanks' balanced salt solution (HBSS) (Thermo Fisher Scientific, Gibco™).
- 1 M HEPES buffer solution (Thermo Fisher Scientific, Gibco).
- Glucose (Thermo Fisher Scientific, Fisher Scientific).
- Trypsin powder (Thermo Fisher Scientific, Gibco™).
- Trypsin inhibitor (Sigma-Aldrich).
- Deoxyribonuclease I (DNase I).
- Sigma-Culture medium (500 ml).
- Dissection medium (500 ml).
- Trypsin (2.5%, 20 ml).
- Trypsin inhibitor (1 mg ml^{-1}, 20 ml).
- DNase (10 mg ml^{-1}, 20 ml).

9.4 Equipment

The equipment list has been adapted from Lian et al. (2016).

- Vented cap T-75 culture flask (Corning).
- Dissection tools.
- Fine scissors (Fine Science Tools).
- Spring scissors (Fine Science Tools).
- Curved standard forceps (Fine Science Tools).
- Fine forceps (Fine Science Tools).
- Centrifuge machine (Eppendorf).
- Hemocytometer.
- Ventilation hood (Thermo Fisher Scientific, Thermo Scientific™).
- CO_2 cell culture incubator (Thermo Fisher Scientific, Thermo Scientific™).
- 37 °C water bath (Thermo Fisher Scientific, Thermo Scientific™).

9.5 Procedure

The procedure described here has been adapted from Lian et al. (2016).

- In the following procedure, three newborn mouse pups were utilized to generate **mixed glial cultures** in two T-75 flasks. In the mixed culture, astrocytes form a confluent cell layer and microglia grow at the bottom and top of the astrocytic layer, respectively. In general, the total primary microglia generated from two T-75 flasks should be enough to seed four 12-well plates at a density of **50,000 cells cm^{-2}**.
- Coat two T-75 culture flasks with 7 ml, 10 μg ml^{-1} PDL for 2 h. Wash the flask bottom with distilled water thrice before use.

  NB: One can coat more flasks than needed while the unused coated flasks can be stored at 4 °C for months. Keep the flask's plastic wrappings to avoid contamination.
- Collect the newborn pups from breeding cages. Keep the pups on a 37 °C hot plate to maintain the body temperature. In the meantime, prepare tools and reagents for the culture experiment. Spray the dissection tools and work space with 75% ethanol. Warm up culture medium in a 37 °C water bath.
- Remove pups from the heating plate. Decapitate and place the heads into a 6 cm Petri dish containing a 5 ml cold dissection medium. Use fine scissors to cut open the scalp along the midline starting posteriorly and ending near the snout. Place one sharp tip of fine forceps beneath the skull at the posterior end of the brain and cut the skull by pushing the end, moving from posterior to anterior. Scoop out the brain using curving forceps and immerse the brains in 5 ml cold dissection media in a new Petri dish.

  NB: Newborn pups have a transparent and soft skull. Using scissors to cut the skull may damage the fragile brain tissue underneath. So, move the scissors gently with caution.
- Put the Petri dish containing the brains under a dissection microscope. Carefully remove the meninges before collecting the cortices and hippocampus. Using three pups is likely to provide six halves. Place three halves per Petri dish with 5 ml dissection medium and grind the tissue into small pieces using spring scissors.
- Transfer the contents of each dish to a 50 ml tube. Wash the dish with a dissection medium to collect any remaining tissue and repeat the transfer. Fill the 50 ml tube to reach a final volume of 30 ml dissection medium.
- Add 1.5 ml, 2.5% trypsin to each tube and incubate in a 37 °C water bath for 15 min. Swirl frequently.
- Add 1.2 ml, 1 mg ml^{-1} trypsin inhibitor, and incubate for 1 min. Add 750 μl, 10 mg ml^{-1} DNase to digest the sticky DNA released from dead cells.
- Centrifuge the tube at 400 rpm for 5 min. Aspirate the supernatant and triturate the pellet with a 5 ml warm culture medium using a 1 ml pipette tip. Transfer the homogenous cell suspension to a 15 ml tube. If chunks of nondissociated tissue persist, let them settle before repeating the trituration and transfer step using a 3 ml medium.
- Centrifuge the 15 ml tubes at 400 rpm for 5 min. Aspirate the supernatant and resuspend the pellet with a 5 ml warm culture medium.
- Perform the cell count using a hemocytometer.

- Plate each tube of cells into one coated T-75 flask at 50,000 cells cm^{-2} density. Add culture medium to reach 15 ml in the flasks. Put seeded flasks into a CO_2 cell culture incubator equipped with 5% CO_2, and 100% humidity at 37 °C.
- Change the culture medium the next day to remove cell debris before making it a routine every 5 days.
- In 5–7 days, astrocytes sediment and grow at the bottom of the flask, as a confluent cell layer. Microglia and some oligodendrocytes grow on top of the astrocytic layer.
- To collect microglia, vigorously tap the flasks on the bench top and collect the floating cells in a conditioned culture medium (no need to change the medium before tapping). The resulting cells are purified microglia.
- Use a hemocytometer to count the floating cells before seeding them at 50,000 cells cm^{-2} in PDL-coated culture vessels. After 2 h, check that the microglia have attached to the bottom under a microscope. Aspirate the medium before replacing it with a fresh culture medium. The microglial cells are ready to use, the very next day.

  NB: ***Most of the steps should be performed in a sterile ventilation hood. Exceptions include the handling of pups, brain dissection, centrifugation, and cell counting. Use sterile tubes and dishes. Spray tools as well as the outside surface of tubes and dishes with 70% ethanol before moving them inside the hood. Except for the 37 °C incubation in the water bath and culture incubator, all steps should be performed at RT, ensuring timid completion for enhanced cell viability.***
- If the mixed glial cells reach confluency but microglial cells are not immediately needed, the mixed glial culture can be passaged. Mixed glial cultures can also be frozen and stored for the long term in a freezing medium composed of DMEM with 20% FBS and 10% DMSO in liquid nitrogen (Fig. 15). When the new passage or recovered frozen cells reach confluency, microglia become capable of growing on top of the confluent cell layer and can be purified by tapping.

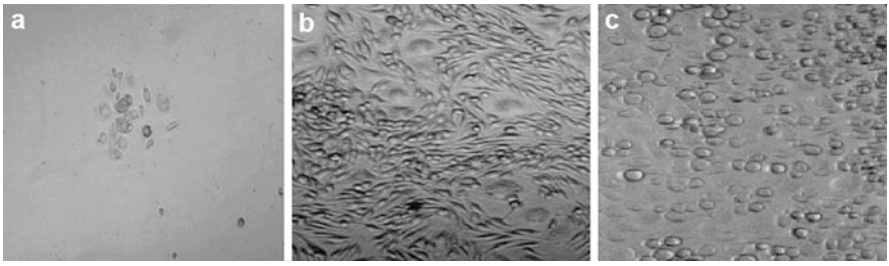

Fig. 15 Typical morphology of cultured microglia cells, illustrating a role of culture media composition on growth intensity, (**a**) mixed retinal culture grown in DMEM, with low cell counts, (**b**) cells grown in nutrient mixture F12 and glutamax supplemented DMEM, showing an increased yield, (**c**) a fraction of cells acquired the spherical morphology on being supplemented with F12 and glutamax (Devarajan et al. 2014)

- Astrocytes and microglia grow more vigorously than oligodendrocytes in the culture conditions. On tapping the mixed glial culture, the collected floating cells may exhibit some oligodendrocytes. However, microglial cells have much stronger attaching capability than oligodendrocytes. After seeding the floating cells, microglia get attached to the culture vessel bottom much more efficiently than oligodendrocytes. On 2 h of seeding before adding the fresh medium, aspiration of the old medium should be done, which would remove the unattached contaminating oligodendrocytes (Galatro et al. 2017; Bohlen et al. 2019).

9.6 Composition of Medium and Buffers

Culture Medium (500 ml)
450 ml DMEM.
50 ml FBS.
Optionally, one may add 5 ml penicillin/streptomycin (PEN/STREP).
Filter and store at 4 °C.

Dissociation Medium (500 ml)
450 ml, 1× HBSS.
5 ml, 1 M HEPES.
3 g glucose powder.
5 ml PEN/STREP solution.
Filter and store at 4 °C.

2.5% Trypsin (20 ml)
Dissolve 0.5 g trypsin powder in 20 ml HBSS.
Filter, aliquot, and store at −20 °C.

1 mg ml^{-1} Trypsin Inhibitor (20 ml)
Dissolve 0.02 g (20 mg) trypsin inhibitor in 20 ml HBSS.
Filter, aliquot, and store at −20 °C.

10 mg ml^{-1} DNase (20 ml)
Dissolve 0.2 g DNase I powder in 20 ml HBSS.
Filter, aliquot, and store at −20 °C.

10 Isolation and Culture of Neuronal Cell Lines

In this section, culturing of two neuronal cell lines, PC-12 and HEK-293 cells, has been discussed. The following paragraphs discuss the culture of these two neuronal cell lines.

10.1 Culture of PC-12 Cells

10.1.1 Origin of PC-12 Cells

The adrenal phaeochromocytoma (PC-12) cell line was derived from a transplantable male rat (*Rattus norvegicus*) pheochromocytoma by Greene and Tischler (1976). This indefinitely cultured (immortal) cell line was developed in parallel to the adrenal chromaffin cell model because of its extreme versatility for pharmacological manipulation, ease of culture, and significant awareness, vis-à-vis proliferation, and differentiation. For example, PC-12 cells resemble the phenotype of sympathetic ganglion neurons on being differentiated with Nerve Growth Factor (NGF). The quality of cultured PC-12 cells provides an advantage despite their smaller vesicles and quantal size, holding an average of merely 1.9 10^{-19} moles of released neurotransmitters. The vesicles hold catecholamines, mostly dopamine besides the limiting extents of norepinephrine, releasing these neurotransmitters to give rise to spikes because of current changes. The PC-12 cell line provides significant information about protein functioning paving way for the vesicle fusion. This cell line has been used to understand the role of synaptotagmin in vesicle-cell membrane fusion.

10.2 Karyotype of PC-12 Cells

It was derived from a transplantable male rat (*Rattus norvegicus*) pheochromocytoma, comprising 40 chromosomes and 38 autosomes including XY.

10.3 Phenotype and Morphological Characteristics of PC-12 Cells

Tissue Type
Adrenal gland (pheochromocytoma of male rat)
Cell Type
This cell line originates from chromaffin cells of the adrenal medulla.
Morphology and Shape
Prevail as adherent culture and as irregular morphology. Suspension state PC-12 cells have a round shape and may form aggregates (Fig. 16).
Diameter
The diameter of this cell line is typically within (6–14) μm.

10.4 Marker Protein(s) Expression in PC-12 Cells

- Nerve growth factor (NGF).
- Genes of neurotransmitters such as catecholamine, dopamine, and norepinephrine.
- Null capacity to synthesize epinephrine.

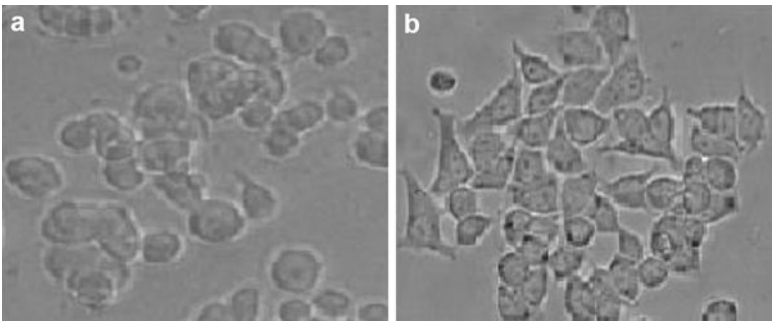

Fig. 16 Morphology of PC-12 cells grown in (**a**) suspension/loosely adherent and (**b**) adherent mode, on RPMI-1640 medium, supplemented with 10% inactivated horse serum and 5% fetal bovine serum

- Express various neuron cell-specific markers such as neurofilament and β-3 tubulin.

10.5 Tumorigenicity of PC-12 Cells

The cell line is claimed to be tumorigenic.

10.6 Culture of PC-12 Cells

In various scientific papers, several PC-12 cell lines have been reported. The ATCC records enlist two PC-12 cell lines. One is the traditional PC-12 cells (**PC-12, ATCC CRL-1721**). This cell line grows as a suspension culture, forming small, irregular, and floating clusters or as few scattered, lightly attached cells. The tendency of forming a cluster of cells is the characteristic of this cell line. The other PC-12 cell line described by ATCC is **PC-12 Adh (ATCC CRL-1721.1**). This cell line grows as an adherent culture with a high growth rate. The adherent cell line was generated by repeated culture in the Corning's CellBIND flasks.

ATCC experiments on neurite outgrowth in response to NGF treatment (0.2 μg ml^{-1}) on collagen-coated plates revealed the development of new neurites on 3 days of NGF incubation.

10.7 Suitable Culture Containers

25 or 75 mm (T-75) flasks.

10.8 Preparation of Culture Medium

The base medium for this cell line is the ATCC-formulated RPMI-1640 medium. To make the complete growth medium, add heat-inactivated 10% horse serum and 5% fetal bovine serum to the base medium.

Several studies also cultured PC-12 cells in complete Dulbecco's Modified Eagle's Medium (C-DMEM), having 10 g L^{-1} glucose, 5% iron-supplemented calf serum, 5% horse serum, and 1% PEN/STREP in a 5% CO_2 atmosphere, as the prominent constituents. Neurons grow well in the 7.2–7.5 pH range. Phenol red is commonly used as a pH indicator.

10.9 Culture Process of PC-12 Cells

Cells grow well in 95% air, 5% CO_2, and at 37 °C.

NB: The traditional PC-12 cells tend to form aggregates. It is observed that in the following aggregation of PC-12 cells, there are alterations of some of the physiological parameters of the cell including loss of secretary responsiveness. Therefore, it is recommended to extensively triturate the cells before replating or reseeding.

Some reports claim that to undergo neuronal differentiation in response to NGF, PC-12 cells must be seeded on collagen IV-coated dishes.

Others claim to coat plates or flasks with poly-L-Lysine followed by seeding cells. Use NGF 50–100 ng ml^{-1} for 8 days from which the differentiated PC-12 cells can be obtained. For confirmation, use mature neuronal markers such as neurofilament and β-3 tubulin to monitor their expression. For differentiation, it is recommended to use RPMI 1640 with 1% horse serum but not fetal bovine serum with antibiotic and mycotic.

10.10 Harvesting and Subculturing of PC-12 Cells

- Allow the cells to grow 70–90% confluence.
- Rinse the cells with Hanks' balanced salt solution (**HBSS**) and 10 mM HEPES.
- Add 5 ml, 0.53 mM EDTA in a 75 cm^2 T-flask and incubate for 5 min in a 37 °C incubator.
- Agitate the flask to dislodge the cells.
- Now triturate the cells again and again (at least ten times) either with a 5 ml pipette fitted with a 1000-ml tip or gently aspirate the cell suspension with a new 20 ml syringe outfitted with a 22 g (1½ inches) needle. This will break up the cell aggregate.
- Now take a 15 ml tube and transfer the cell suspension into it.
- Centrifuge at 300 rpm for 5 min at 4 °C.
- Count the number of cells.
- Take a new cell culture container.

- Seed a 75 mm flask with 5×10^5–1×10^6 viable cells ml^{-1} or use 1:2–1:4 subcultivation.
- Incubate the culture vessels in a CO_2 incubator at 37 °C.
- When the cell density reaches within 2–4 $\times$ 10^6 viable cells ml^{-1}, the culture is ready for further subculturing.
- Medium Renewal: Every 2–3 days.

10.11 Growth Characteristics of PC-12 Cells

Suspension culture grows in clusters, having few scattered and lightly attached cells. The adherent culture (homogeneously attached to the culture vessel surface) exhibits an irregular morphology.

10.12 Population Doubling Time

48 h

10.13 Freezing And Thawing of PC-12 Cells

Complete growth medium supplemented with 10% (v/v) DMSO. The preferred storage condition comprises the liquid nitrogen vapor phase.

10.14 Usage of PC-12 Cells

- Some of the classical neurological studies employing neuronal cell lines were completed using the PC-12 cell line. For example, it was observed that treatment of PC-12 cells with neuronal growth factor (NGF) leads to arrested proliferation and neurite outgrowth. This classical experimental observation demonstrated the ability of the PC-12 cells to acquire the sympathetic neurons on being fed with NGF.
- The various neurotransmitters released by the neuronal cells are acetylcholine, epinephrine/dopamine, serotonin, gamma-aminobutyric acid (GABA), etc. This cell line is used to understand the basic biology of the neuronal cells including neuroendocrine secretion. It helps to understand the specific culture conditions and the growth environment that affects the neurosecretory pathways. Based on the results of various experimental studies, it is claimed that the PC-12 cell line can provide an excellent model for studying chemical disruption associated with neuronal differentiation, synthesis, storage, and release of neurotransmitters.
- This cell line is also utilized to create artificial nervous system models that are utilized for neurodegeneration studies.

- The biocompatibility of high-density electrode arrays is performed using this cell line.
- Finally, this cell line also finds utility as a suitable transfection host model.

10.15 Biosafety of PC-12 Cells

Level 1 only.

11 Origin and Culture of HEK-293 Cells

- In 1973, **Dr. *Alex Van der Eb's*** laboratory from the *University of Leiden* generated a cell line from human embryonic kidney (HEK) cells using an aborted normal (without any disease) human fetus via transformation with sheared adenovirus 5 DNA.
- In this transformation, a 4.5 kb viral DNA was incorporated into chromosome 19 of the HEK cells.
- Thus, HEK terminology was given due to belonging to human embryonic kidney cells and 293 is an arbitrary number or the number of optimizing experiments in *Alex Van der Eb's* laboratory for a viable culturing.
- HEK-293-T is a human cell line, derived from the HEK-293 cell line carrying a mutant version of SV40 large T antigen (Vaccines and Related Biological Products Advisory Committee 2001).

11.1 Genomic and Proteomic Dynamics of HEK-293 Cells and Their Usage in Neurological Studies

- Several gene expression and transcriptomics data indicate that HEK-293 cells originate from the adrenal gland which is well known for its proximal position to the kidneys. It is established that the adrenal medulla has a neural crest origin. This supports the expression of several neuron-specific genes in HEK-293 cells. This cell line expresses the notable early neuronal differentiation markers such as vimentin and nestin besides the keratin 8 and keratin 18 expression. Study results of a group of scientists showed the adenovirus-transformed HEK cell lines are similar to that of a typical early differentiating neuron or neuronal stem cells (Shaw et al. 2002).
- It is noticed that corticotropin-releasing factor type I receptors that are highly released by the brain and pituitary gland are also expressed by this cell line. This receptor molecule causes an intracellular increment of cAMP (Dautzenberg et al. 2000).
- Rigorous microarray analysis showed that this cell line also expresses thyrotropin-releasing hormone receptor, somatostatin receptor subtype SSTR2, sphingosine-1-phosphate receptors, β2-adrenergic receptors, muscarinic acetylcholine receptors, and P2Y1 and P2Y2 (family of purinergic G protein-coupled receptors). The expression of these marker proteins confirms the neurological origin of this cell line.
- One of the important characteristics of neuronal cells is to examine the activity of voltage-gated ion channels. Similar to other neuronal cells, HEK-293 cells

express several endogenous voltage-activated ion channels and regulate their activation (Shaw et al. 2002; Lin et al. 2014).

Thus, there are many reasons for the wide use of HEK-293 cells for neurological analysis, briefly summarized in the following points:

- The first and most important reason is that the HEK-293 cells are easy to handle, capable of rapid growth, and can be readily transfected besides being amenable to stringent quantitative assessments, and therefore, can be used as cell lines for general biological studies.
- Secondly, the HEK-293 cells provide a reasonable approximation for addressing manifold basic biology concerns about neuron culturing.
- Thirdly, HEK-293 cells are devoid of several key proteins that play critical roles in the biology of neurons, such as ion channels, receptors, enzymes, chaperones, and so on. As such, HEK-293 cells provide a perfect low "noise" natural model for studying the biology and physiology of these proteins.
- Finally, this cell line is utilized for understanding and knowing about the pharmacological and biophysical characteristics of ion channels and receptors by various procedures, including the patch-clamp technique.

11.2 Karyotype and Short Tandem Repeat Sequence Driven Identification of HEK-293 Cells

- HEK-293 cells represent a hypotriploid human cell line.
- The modal chromosome number of these cells is 64.
- Chromosomal abnormalities include three copies of the X chromosome and four copies of the 17th and 22nd chromosomes.
- These cells exhibit multiple X chromosomes and lack any Y chromosome-derived sequence, suggesting their association with a female fetal source.
- The use of short tandem repeat markers has been recommended for cell line authentication and confirmatory screening of HEK-293 cells. These methods are currently being used to identify human cell lines.

Some prominent characteristic short tandem repeat sequences used to identify HEK-293 cells are as follows:

Amelogenin: X
CSF1PO: 11,12
D13S317: 12,14
D16S539: 9,13
D5S818: 8,9
D7S820: 11,12
THO1: 7,9.3

TPOX: 11
vWA: 16,19

11.3 Phenotype and Morphological Characteristics of HEK-293 Cells

Tissue Type
Human embryonic kidney.

Cell Type
This cell line exhibits a close resemblance with adrenal cells rather than the typical kidney epithelial cells.

Morphology
Epithelial (Fig. 17).

Shape
Round.
Cells have a large nucleus, occupying ~ two-thirds of the entire volume.

Diameter
Within (15–20) μm.

11.4 Marker Proteins Expression

Various proteins are expressed by this cell line, prominent amongst which are vitronectin, Nestin, CD133, Notch1, and nerve growth factor receptor. The other proteins expressed by the HEK-293 cell line are Snai2, Sox9, Sox10, Phox2b, and Ascl1.

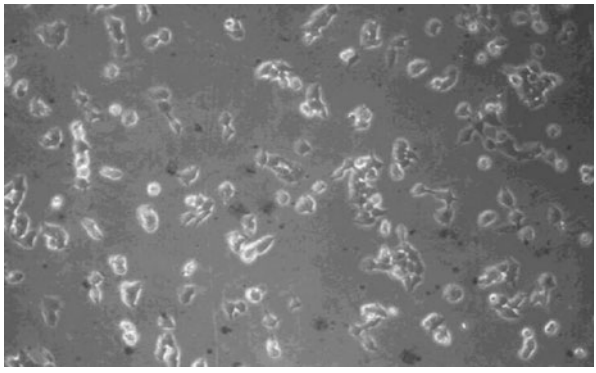

Fig. 17 An inverted microscopic view of cultured HEK-293 cells (Ghosh et al. 2011)

11.5 Tumorigenicity of HEK-293 Cells

- Passage level-dependent tumorigenicity, with 100% expression for >65 passage number.
- No tumor is induced for a <52 passage number.
- These observations/conclusions are the findings of studies on nude mice models.

11.6 Culture of HEK-293 Cells

Suitable Culture Containers
T-75 flasks are preferred, although other cell culture vessels can also be used.

Preparation of Cell Culture Medium
Eagle's Minimum Essential Medium (EMEM, ATCC formulated) with 10% FBS (Liste-Calleja et al. 2013).

NB: In general, the cells do not need any additives for healthy growth. Cells also grow well in Dulbecco's Modified Eagle medium (DMEM), having high glucose content (4.5–5 g L^{-1}).

11.7 Culture Process of HEK-293 Cells

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one HEK-293 cryovial containing 1×10^6 cells ml^{-1} and aseptically transfer it to the 75 mm flask.
- Incubate the flask in an incubator equipped with 5% CO_2, and 95% moisture at 37 °C.
- Change the medium after 24 h to remove DMSO and floating cells.
- This is a very rapidly growing cell line.
- Renew the medium two to three times a week, keeping the cell count between 1×10^5 and 3×10^5 cells ml^{-1}.
- Do not allow cell concentration to exceed 1×10^6 cells ml^{-1}.
- The approximate cell number to attain 100% confluency for this cell line in a T-75 flask is 1×10^7 cells.

11.8 Harvesting and Subculturing of HEK-293 Cells

- Cells attain a splitting number within 1×10^5–3×10^5/ml in ~2–3 days.
- HEK-293 cells can easily be detached by washing out with a growth medium.
- There is no need to trypsinize. Just take a 10 ml pipette and wash the cells out by pipetting up and down in the cell culture medium.
- Harvest and perform low-speed centrifugation.

- Aspirate the supernatant, resuspend with cell culture medium, and split at 1:4–1:8 proportion.

11.9 Growth Characteristics of HEK-293 Cells

Culture Properties
Loosely adherent cells, but can also grow in suspension mode. The cells can be removed while being inside the culture flask without adding trypsin-EDTA.

Growth Pattern
Continuous growth (immortal cell line).

Population Doubling Time
20–24 h. Doubling time depends on the medium composition.

Freezing and Thawing of HEK-293 Cells
Cells can be stored as a stock in liquid nitrogen at 2–5 $\times$ 10^6 cells ml^{-1} in a growth medium containing 5–10% DMSO. Use the standard procedure for freezing and thawing.

11.10 Usage of HEK-293 Cells

- The growth and maintenance of this cell line are relatively simple with high reproducibility as compared to other cell lines. Thus, this cell line is preferable to other less robust and slow-growing cell lines.
- HEK-293 is one of the easiest cell lines to transfect with various vectors including adenovirus. This cell line can be used for the proliferation of adenovirus vectors. Of note, while the cell line contains several adenovirus genes, but lacks the hazardous E1 and E2 genes.
- These cells are commonly used for protein expression and the production of recombinant retroviruses. For example, a recombinant activated protein C was synthesized, isolated, and later, screened as a novel protein for the sepsis treatment.
- This cell line is being used to examine the interactions between different proteins and drugs. The befitting example of this includes the screening of effects of drugs on sodium channels using this cell line.
- Around the year 2005, a HEK-293 cell line variant, called **HEK 293T** was developed. **SV40 Large T antigen** is present in this cell line. Of note, the transfected SV40-containing plasmids can undergo episomal replication, enhancing the concentration of recombinant protein/retroviral production (Thomas and Smart 2005).

11.11 Biosafety of HEK-293 Cells

Level 2 (cells contain adenovirus).

12 Conclusions

This chapter describes the isolation and culture of neuronal cells, supporting cells, and neuronal cell lines. The supporting cells are large in number and continuously divide throughout a mammal's life. While adult neurons do not undergo any division, neuronal stem cells continue to divide throughout a mammal's life. However, only certain brain regions such as the hippocampus, striatum, and cerebellum contain NSCs. The number of NSCs is very few not only concerning neuronal cells but also compared to supporting cells. So, isolation and culture of neurons from the brain is a difficult task. Under the prevailing conditions, the culture of immortal neuronal cell lines such as PC-12 cells and HEK-293 cells may provide some clues about the structural and functional characteristics, although immortal cell lines considerably differ in genotype and phenotype from their normal counterparts. This chapter enriches the knowledge about the isolation and culture process of NSCs from various regions of the brain, the culture of supporting cells, and neuronal cell lines namely PC-12 cells and HEK-293 cells.

13 Cross-References

- ▶ Culture of Continuous Cell Lines
- ▶ Isolation and Purification of Various Mammalian Cells: Single Cell Isolation
- ▶ Isolation and Primary Culture of Various Mammalian Cells
- ▶ Primary Culture of Immunological Cells
- ▶ Stem Cell Culture and Its Applications

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Culture of Continuous Cell Lines

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Contents

Abstract

This chapter describes the culture of various continuous cell lines. Continuous cell lines are generated either due to specific genetic or epigenetic alterations in the genome of certain cells. The reasons for genetic alterations are the

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_11-1

manifestation of spontaneous or induced mutations via radiations and chemical or biological agents such as viruses. In the experimental laboratory conditions, continuous cell lines can be generated by a viral infection, biochemical agents, or radiations. This chapter discusses the culture of major mammalian continuous/cancer cell lines, including breast cancer cell line (**MCF-7** and **MDA-MB-231** cell), ovarian cancer cell line (**CHO** cells), prostate cancer cell line (**LNCaP** and **PC3** cells), cancer cell lines originating from the female cervix (**HeLa** cells), lung cancer cell lines (**A549** and **BEAS-2B** cells), kidney cancer cell lines (**HEK-293** and **VERO** cells), liver cancer cell lines (**HepG2** cells), and blood cancer cell lines (**JURKAT** and **RAW** cells). While some of the abovementioned cancer cell lines are isolated directly from various cancer patients, others are experimentally generated in the laboratory. The knowledge gained from this chapter would help to understand the culture and regular maintenance of the abovementioned continuous cell lines and in the development of various anticancer agents.

Keywords

Culture of Continuous /Cancer Cell Lines · MCF-7 Breast Cancer Cell Line · MDA-MB-231 Breast Cancer Cell Line · CHO Ovarian Cancer Cell Line · LNCaP Prostate Cancer Cell Line · PC3 Prostate Cancer Cell Line · HeLa Cervix Cancer Cell Line · A549 Lung Cancer Cell Line · BEAS-2B Lung Cancer Cell Lines · HEK-293 Kidney Cancer Cell Lines · VERO Kidney Cancer Cell Line · HepG2 Liver Cancer Cell Line · JURKAT Blood Cancer Cell Lines · RAW Blood Cancer Cell Lines

1 Introduction

Under normal physiological conditions after a certain number of divisions, normal mammalian cells lose the capacity for division and eventually perish due to aging or senescence. Thus, the normal mammalian cells are considered **mortal, with finite cell divisions.** This is due to the shortening of the telomere on each successive division, known as the **Hayflick effect**. Depending upon the cell type, most mammalian cells die due to a lack of telomere and other related factors after about **50–100 divisions**.

However, a normal mortal cell may be **transformed** into an immortal cell. The immortal cells are naturally developed due to spontaneous exposure to various intrinsic factors (e.g., free radicals/hormones/DNA tautomerism) and/or extrinsic factors (e.g., radiations, chemicals, viruses) that change or transform a normal mortal cell into an immortal one. Genetic mutations are the reasons for this transformation. Additionally, a normal mortal cell can be converted into an immortal one by treatment with chemicals or radiations or via transfection with specific oncogenic viruses such as Simian virus 40 (SV40). Such transformed immortal cells maintain the telomere length with the aid of **telomerase,** via adding the telomere repeat sequences to the 3′ end of DNA strands. The generated immortal cells divide indefinitely in an in vitro culture, subject to the nutrition provision. These cells

that divide indefinitely are also called **continuous cell lines**. Besides genetic mutation, epigenetic changes such as DNA methylation or acetylation also may result in continuous cell division (Todaro et al. 1964).

After having accumulated adequate mutations, a continuous cell line may generate tumors or cancers. With the further accumulation of irreparable mutations, these tumors may acquire the **capacity for invasion** (forceful occupation of other cells' space) and **metastasis** (movement to other body regions through circulation followed by attachment and growth in a new organ(s) other than of their origin). Thus, initially benign tumor cells may gain the invasion capacity and become capable of metastasis as malignant or cancer cells (Irfan Maqsood et al. 2013).

This chapter describes the basic characteristics and culture of various continuous/cancer cell lines derived either in a laboratory either via normal to immortal cell transformation through viral induction or via chemicals/other agents (e.g., radiation) or on being directly isolated from various tumors/cancers. The continuous cell lines discussed in this chapter are breast cancer lines (**MCF-7** and **MDA-MB-231** cells), ovarian cancer line (**CHO** cells), the prostate cancer line (**LNcaP** and **PC3** cells), cancer cell lines originating from the female cervix (**HeLa** cells), lungcancer cell lines (**A549** and **BEAS-2B** cells), kidney cancer lines (**HEK-293** and **VERO** cells), the liver cancer line (**HepG2** cells), and blood cancer lines (**JURKAT** and **RAW** cells).

Each continuous cell line is discussed *vis-a-vis* origin, karyotype, short tandem repeat (**STR**) sequences (to confirm the originality), phenotype and morphological characteristics, marker protein(s) expression, tumorigenicity, culturing requirements, growth pattern, harvesting and subculturing, freezing and thawing, usage, and biosafety. References are amicably included. This chapter also explains the general culturing procedures, being widely used at present for the abovementioned cell lines.

2 Basic Hallmarks of Immortal Continuous Culture Cells

Cancer cells are said to be immortal or continuously dividing cells. In 2000, ***Douglas Hanahan*** and ***Robert Weinberg,*** through a publication in *Cell,* described the following six characteristic traits of cancer cells:

1. Growth signals self-sufficiency
2. Antigrowth signals insensitivity
3. Apoptosis evasion
4. Replicating potential unlimited
5. Sustained angiogenesis
6. Tissue invasion and metastasis

In 2011, through another publication in **Cell*****, the same authors added four additional characteristics as follows***:

7. Abnormal metabolic pathways
8. Immune system evasion

9. Instability in the genom
10. Inflammation

At present, these 10 characteristics are widely recognized as "Hallmarks of Cancer Cells" (Hanahan and Weinberg 2000, 2011).

3 Sources of Immortal Continuous Culture Cells

The following are the examples of culturing continuous cells:

- Isolation of continuous/cancer cells directly from cancer-affected organs of human/animal subjects. Example: Michigan Cancer Foundation (**MCF**)-7 breast cancer cells were isolated from the pleural effusion of a 69-year-old Caucasian woman who was affected by breast adenocarcinoma. Similarly, a large number of cancer cell lines were originally isolated from the cancer patients.
- Transformation of mortal cells to continuous immortal cells via exposure to chemical carcinogens, radiation, or viruses. Example: human embryonic kidney (**HEK**)-293 cells, isolated from an aborted fetus and transformed using **adenovirus**.
- Induced telomerase expression for a continuous telomere availability during the mammalian cell culture. Normally eukaryotic cells have a limited number of divisions due to the shortening of telomere after each division. This is known as the **Hayflick effect.**
- Generation by **hybridoma technology**, where antibody-producing B cell-derived plasma cells are fused with a myeloma cell (B cell cancer). The hybrid cells thus produced are utilized for monoclonal antibody production with continuous division.
- American Type Culture Collection **(ATCC)** is the largest reservoir of various cell lines, including immortal continuous/cancer cell lines. Additionally, throughout the world, there are other sources of cell lines, including the European Collection of Cell Cultures (**ECCC**) and various commercial companies (e.g., Sigma).
- ATCC and other organizations have their own media compositions and culture conditions for different cells.
- In some cases, researchers have altered these culture protocols for the best results of their research.
- Various research forums like **Research Gate, LinkedIn,** etc., extensively discuss the pros and cons of different cell culture protocols.
- Thus, the culture protocols mentioned in this chapter may reflect various corrections/modifications implemented by various researchers or companies over several years of research experience.

4 Culture of Immortal Continuous Culture Cells

As mentioned above, immortal continuous/cancer cells originate from various organs of the human body/other mammalian bodies. Here, the culture of various continuous cell lines originating from **mammalian breast, ovary, endometrium, cervix, prostate, liver, lung, kidney, and blood** is discussed.

4.1 The Culture of Immortal Continuous Cells Originating from the Mammalian Breast: MCF-7 Cells and MDA-MB-231 Cells

Culturing of these two immortal breast cell lines is done as overleaf:

Here is the culture procedure for both of them:

4.1.1 The Culture of MCF-7 Breast Cancer Cells

Origin of MCF-7 Cells

In 1973, ***Herbert Soule*** and coworkers at Michigan Cancer Foundation in Detroit, USA, isolated and established a cell line from the pleural effusion of a 69-year-old Caucasian woman who was afflicted with breast adenocarcinoma. They named these cells MCF-7 after the organization. The reason for using the number 7 is not known (Soule HD et al. 1973).

Karyotype of MCF-7 Cells

- The MCF-7 cells originally contained 85 chromosomes.
- The present MCF-7 cells have 69. This change in chromosome number is due to thousands of culture passages.
- Of note, there are genetic discrepancies between the MCF-7 cell lines obtained from the Michigan Cancer Foundation (the original discoverer) and the ATCC. This may be due to continued culture for so many generations.

Identification by Short Tandem Repeat Sequence

The use of **short tandem repeat markers** has been recommended for cell line authentication and identification of MCF-7 cells. The approach is currently being used to identify numerous human cell lines (Masters 2001).

Some of these markers are as follows:

Amelogenin: X
CSF1PO: 10
D13S317: 11
D16S539: 11, 1
D5S818: 11, 12.
D7S820: 8, 9
THO1: 6

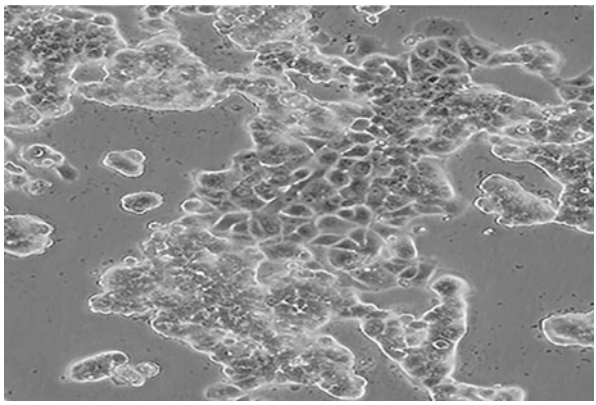

Fig. 1 Cultured MCF-7 cell line, as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/crl-3435)

TPOX: 9, 1
vWA: 14,15

Phenotype and Morphological Characteristics of MCF-7 Cells

- **Tissue type**
  Human luminal A breast cancer cells. The metastatic cells were isolated from pleural effusion.
- **Cell type**
  Epithelial.
- **Morphology**
  Retain epithelial morphology (Fig. 1).
- **Shape**
  When grown in vitro *(2D),* the cell line is capable of forming domes.
- **Diameter**
  (15–17) μm.

Marker Proteins Expression in MCF-7 Cells

- Express estrogen receptors (**ERs**).
- Express progesterone receptors (**PRs**).
- Do not express the **Her2/Neu** receptor.
- Do not express **caspase 3** protein.
- 95% of MCF-7 cells express **EpCAM.**

NB: Approximately (70–80) % of all breast cancers are positive for ERs and PRs (Brandes and Hermonat 1983).

ER and PR-positive MCF-7 cells are responsive to estrogen or progesterone treatment.

These cells are also responsive to selective estrogen receptor modulators (**SERMs**) (e.g., tamoxifen), selective estrogen receptor down-regulators (**SERDs**)

(e.g., ICI182780 or fulvestrant), and **aromatase** (the testosterone to estrogen-converting enzyme) inhibitors such as anastrozole.

Tumorigenicity of MCF-7 Cells

Injection of 1×10^5–1×10^7 MCF-7 cells in the **nude mice** (mice lacking thymus or thymectomized) breast pad generates breast cancer.

NB: Several reports suggest that estrogen supplementation is essential for the tumorigenic transformation of MCF-7 cells. Tumor necrosis factor-alpha (TNFα) inhibits the growth of MCF-7 breast cancer cells.

Culture of MCF-7 Cells

Suitable Culture Containers

T-75 flasks. 100 mm Petri plate or other culture containers can be used.

Preparation of Cell Culture Medium

- Dulbecco's Modified Eagle's Medium (**DMEM**) with high glucose (4.5 g/liter), 10% FBS, 100 U/ml penicillin G, 100 μM/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B.
- For a healthy and rapid growth rate, the following materials should be added to the complete cell culture medium: (1–2) mM L-glutamine, 0.01 mg/ml insulin, and 1 mM sodium pyruvate.

NB: MCF-7 cells generally grow in clusters.

- The use of antibiotics and antimycotics in cell culture media is optional.
- The culture medium pH should be slightly alkaline, that is, around 7.4–7.5. Note that while human cell cytoplasm pH is 7.2, human blood pH is 7.4. Regular inspection of color changes *vis-a-vis* pH indicators (phenol red) in the cell culture medium is essential.
- Since metabolic products of cells (e.g., lactic acid) decrease the culture pH, sodium bicarbonate is frequently used. However, caution should be taken because the high concentration of sodium bicarbonate could result in excessive alkalinity.
- To diminish the toxic action of accumulated metabolites on the actively growing cells, regular replacement of the cell culture medium is necessary.

Culture Process of MCF-7 Cells

- Take a 75 mm mammalian cell culture-grade flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one MCF-7 cryovial containing 1×10^6 cells/ml and aseptically transfer to.
- 75 mm flask.
- Incubate the flask in a CO_2 incubator with 5% CO_2, and 95% humidity, maintained at 37 °C.
- The next day, change the medium to remove DMSO and floating cells.

- Replace the old culture medium with a new cell culture medium, every 2–3 days.
- Within 5–6 days, 80–90% of the 75 mm flask culture surface area will be full and ready for subculturing.
- It may take only a short time to achieve confluency with the abovementioned additives such as insulin, on being added to the cell culture medium.
- At this time, the 75 mm flask may contain 8–10 million MCF-7 cells.

NB: A 75 mm flask is also called a T-75 flask.

Harvesting and Subculturing of MCF-7 Cells

- Allow MCF-7 cells to reach a confluency of approximately 80–90%.
- Remove the cell culture medium.
- Rinse the cell with 1× PBS.
- Now detach the cells from the culture flask by adding warm (37 °C) trypsin (0.25 (w/v)%) and EDTA (0.53 mM).
- Observe cells under an inverted microscope. Dispersal should happen within (3–5) min.
- In case detachment takes a longer time, the culture flask with trypsin may be incubated at 37 °C for a few minutes.
- Additionally, one may need to mildly shake the culture vessel for cell detachment from the walls. However, there is a chance of cell clumping, particularly MCF-7 cells tend to clump during cell harvesting.
- Following detachment, the trypsin is neutralized immediately by adding 10 ml of complete medium to the flask. The used-up medium may be aseptically collected before trypsinization if this medium does not contain dead cells and is used for neutralization.
- To pellet down the cells, centrifuge for 5 min at 1000 rpm, 4 °C.
- Remove supernatant (trypsin/growth medium) from the tube.
- Add 10 ml fresh medium to resuspend the cells.
- Split the cells at a **1:3 or 1:6** sub-cultivation ratio.

Growth Characteristics of MCF-7 Cells

- **Culture propertie**
  Loosely adherent in nature.
- **Growth pattern**
  Continuous (immortal cell line).
- **Population doubling time**
  Freshly growing 90% confluent cells that are just subcultured have ~**24 h** of doubling time.

Freezing and Thawing of MCF-7 Cells

- Prepare a mammalian cell culture-grade freezing medium by adding 10% mammalian cell culture-grade dimethylsulfoxide (DMSO) and a 90 ml complete medium.
- Add 1 ml freezing medium per $\mathbf{1 \times 10^6}$ **cell**s and make a cell suspension.

- Put it into a cryovial.
- Put the cryovials in the liquid nitrogen vapor phase following a standard slow freezing procedure.
- For thawing the cryopreserved cells, switch on the water bath set at 37 °C and allow the temperature to reach 37 °C. Take out the vial from liquid nitrogen and immediately thaw the vial by putting the vial at 37 °C.
- As soon as the vial is thawed, immediately clean the outer surface of the vial in 70% ethanol and septically transfer the vial material into the cell culture medium.

Usage of MCF-7 Cells

MCF-7 cells are and have been used for the following purposes:

- To study in vitro breast cancer pathophysiology.
- For injection into the nude mice, use a breast pad to create a breast cancer animal model.
- To study the effects of estrogens, their various metabolites, and anti-estrogen molecules on breast cancer cells.
- To study the role of estrogen receptors (**ERα and ERβ**) and anti-ER molecules in breast cancer complications.
- To study the mechanism of **tamoxifen** (a competitive inhibitor of estrogen for ER) resistance.
- To study the role of estrogen-related receptors **(ERRs: ERRα, ERRβ, ERRγ**), the members of the nuclear hormone receptor superfamily in various types of cancer complications and drug resistance.

NB: The phenol red color of the cell culture medium indicates estrogenic activity. Therefore, it should be removed from the culture media before treatment with estrogens or estrogenic compounds.

- ***Growth factors, serum, sodium pyruvate, insulin,* etc.*, must be removed from the cell culture medium to observe the effect of estrogen on MCF-7 cells.***
- ***Breast cancer models can also be created by transferring breast cancer tissues from one to another syngeneic mouse.***

Biosafety of MCF-7 Cells

Level 1 only.

4.1.2 The Culture of MDA-MB-231 Breast Cancer Cells

Origin of MDA-MB-231 Cells

- In 1977, ***R. Cailleau*** and colleagues at MD Anderson Cancer Center, Texas, USA, isolated and characterized a metastatic adenocarcinoma cell line from the pleural effusion of a 51-year-old African-American woman (Cailleau R et al. 1974, 1978; Cruciger Q et al. 1976).

- **MDA in the cell line name denotes M.D. Anderson, MB denotes metastatic breast cancer.**
- The number 231 most probably denotes the experiment number of the scientist identifying the line.
- Variants of MDA-MB-231 cells include MDA-MB-453 and MDA-MB-468 and are available.
- Later studies also confirmed the **highly metastatic potential** of this cell line (Price JE et al. 1990).

Karyotype of MDA-MB-231 Cells

- In this cell line, the modal number 64 ranges from 52 to 68, with near triploid range chromosome counts. Therefore, the MDA-MB-231 cell line is well recognized as aneuploidy.
- N8 and N15 (normal chromosomes) are absent.
- The majority of autosomes demarcate the trisomy.
- Along with non-assignable chromosomes, 11 stable rearranged marker chromosomes are noted.
- ***K.L. Satya-Prakash*** *and colleagues* reported many marker chromosomes.
- The MDA-MB-231 cell line harbors five mutations, namely, TP53, BRAF, CDN2A, KRAS, and NF2.

Identification of Short Tandem Repeat Sequence

The use of short tandem repeat markers is recommended for cell line authentication and identity confirmation of MDA-MB-231 cells and is also widely used to identify human cell lines.

The following are the key marker sequences notified:

Amelogenin: X
CSF1PO: 12, 13
D13S317: 13
D16S539: 12
D5S818: 12
D7S820: 8,
THO1: 7, 9.3.
TPOX: 8, 9
vWA: 15,18

Phenotypic and Morphological Characteristics of MDA-MB-231 Cells

- **Tissue type**
  Human breast.
- **Cell typ**
  Epithelial.
- **Morphology.**
  Epithelial.
- **Shape**

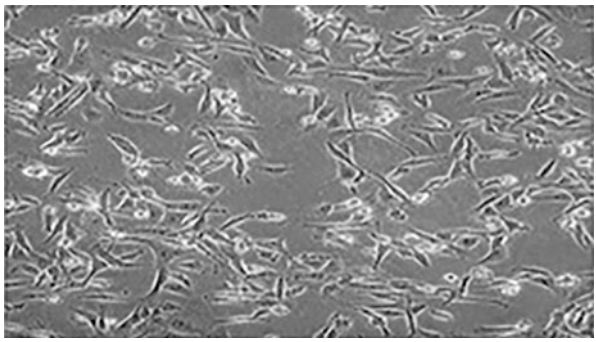

Fig. 2 Cultured MDA-MB-231 cells as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/htb-26)

Spherical to the spindle, with a mesenchymal shape (Fig. 2).

- **Diameter**
  (12.4 ± 2.1) μm.

Marker Proteins Expression in MDA-MB-231 Cells

- Negative for estrogen receptors (**ERs**), progesterone receptors (**PRs**), and **Her2/Neureceptors**. So, it is identified as a true **triple negative.**
- Highly expressed mesenchymal markers including **vimentin** and **moesin.**
- Low expression **of claudin.**
- Low expression of **E-cadherin.**
- Low expression of **EpCAM.**

Tumorigenicity of MDA-MB-231 Cells

- Injection of 1×10^5 viable MDA-MB-231 cells into the mammary/breast fat pad (**MFP**) of **nude mice** revealed a 100% tumor generation probability.
- The subcutaneous injection of **1×10^5 viable MDA-MB-231 cells** into nude mice gives only a 40% chance of tumor generation, and these occur several weeks after mammary fat pad tumor appearance.
- The original MDA-MB-231 cells exhibit **high invasion and metastatic features**.

Culture of MDA-MB-231 Cells

1. **Suitable Culture Containers.**
   T-75 flasks are preferred, and other cell culture vessels can also be used.
2. **Preparation of Cell Culture Medium.**
   DMEM (high glucose, (4.5–5) g/L**)**, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μM streptomycin sulfate, 0.25 μg/ml amphotericin B.

NB: For rapid growth, the following entities should be added to the cell culture medium: 0.01 mg/ml insulin, 1 mM sodium pyruvate, and 2.2 g/ml sodium bicarbonate (for pH adjustment).

- ***The use of antibiotics or antimycotics in the culture media is optional.***
- ***The cell culture medium pH should be 7.2–7.4. Note that while human cell cytoplasm pH is 7.2, human blood pH is 7.4. Regular screening of the color changes in the culture medium is essential.***
- ***Since metabolic products of cells (e.g., lactic acid) decrease the medium pH, sodium bicarbonate is added. However, caution should be taken that a high sodium bicarbonate concentration may make the culture medium too alkaline.***
- ***To diminish the effects of accumulated metabolites in the actively growing cells, regular replacement of the cell culture medium is mandatory.***

3. **Culture Process of MDA-MB-231 Cells.**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one MDA-MB-231 cryovial containing **1.5×10^6 to 2×10^6 cells/ml** and aseptically transfer the same to the 75 mm flask.
- Incubate the flask in a CO_2 incubator with 5% CO_2 and 95% moisture at 37 °C.
- Change the medium the next day to remove DMSO and floating cells.
- Replace old cell culture medium with new cell culture medium every 3 days.
- Within 3–4 days, 80–90% of the 75 mm flask culture surface area will be covered by cells, with readiness for subculturing.
- At this time, the 75 mm flask will contain approximately **10–12 million** MDA-MB-231 cells.

NB: 75 mm flask is also called a T-75 flask.

Harvesting and Subculturing of MDA-MB-231 Cells

- Allow MDA-MB-231 cells to reach a confluency (~2×10^5 cells/cm^2) of approximately 80–90%.
- Remove the cell culture medium.
- Rinse the cell with 1× PBS.
- Now detach the cells from the culture flask by adding warm (37 °C) trypsin (0.25 (w/v)%) and EDTA (0.53 mM).
- Observe cells under an inverted microscope. Dispersal should happen within (3–5) min.
- In case detachment takes a longer time, the culture flask with trypsin may be incubated at 37 °C for a few minutes.
- Additionally, one may need to mildly shake the culture vessel for cell detachment from the walls. However, there is a chance of cell clumping.
- Following detachment, the trypsin will be neutralized immediately with 10 ml complete medium to the flask. The used-up medium may be aseptically collected before trypsinization if this medium does not contain dead cells and is used for neutralization of trypsin.
- To pellet down the cells, centrifuge for 5 min at 1000 rpm, 4 °C.
- Remove supernatant (trypsin/growth medium) from the tube.
- Add 10 ml fresh medium to resuspend the cells.

- Split the cells into 1:4 or 1:5 proportions.
- Generally, a split ratio of 1:4 to 1:5 or a seeding density of (4×10^4 to 5×10^4) cells/cm^2 is used while subculturing MDA-MB-231 cells.

Growth Characteristics of MDA-MB-231 Cells

- **Culture properties**
  Adherent.
- **Growth pattern**
  Continuous culture (immortal cell line).
- **Population doubling time**
  Freshly growing 90% confluent cells that are just subcultured require nearly **24 h** to double themselves.

Freezing and Thawing of MDA-MB-231 Cells

- MDA-MB-231 cells can be cryopreserved in complete growth medium supplemented with 10% (v/v) mammalian cell culture-grade DMSO at between 1.5×10^6 cells/ml and 3×10^6 cells/ml (average 2×10^6 cells/ml).
- Store frozen cell vials in the liquid nitrogen vapor phase after following a standard slow freezing procedure.

Usage of MDA-MB-231 Cells

- Estrogen-insensitive breast cancer cell line (around 15% of all breast cancers), is appropriate to check the effects of non-estrogen molecules (e.g., anti-androgen molecules) as chemotherapeutic agents.
- Ideal cell line to screen the efficacy of combination drug therapy against breast cancer.
- Ideal cells to examine breast cancer invasion and metastasis.
- Useful in deciphering molecular mechanisms of drug resistance.
- Table 1 outlines the distinctions in culture characteristics of MCF-7 and MDA-MB-231 breast cancer cell lines for their explicit experimental recognition.

Table 1 Major differences in MCF-7 and MDA-MB-231 breast cancer cells

MCF-7 cells	MDA-MB-231 cells
Estrogen receptor (**ER**) positive Estrogen-sensitive cell line	Estrogen receptors (**ER**) are negative Estrogen-insensitive cell line
Progesterone receptor **(PR**) positive Progesterone-sensitive cell line	Progesterone receptor (**PR**) negative Progesterone-insensitive cell line
HER-2 negative	**HER-2** negative
95% of MCF-7 cells express **EpCAM**	Very low-level **EpCAM** expression
Do not express **caspase 3**	Express **caspase 3**
Low-level expression of mesenchymal markers such as **vimentin** and **moesin**	High expressions of mesenchymal markers such as **vimentin** and **moesin**
Cells may grow in a cluster	Cells do not grow in clusters
Very low invasion and metastasis rate	High rate of **invasion** and **metastasis**

Biosafety of MDA-MB-231 Cells
Level 1 only.

4.2 The Culture of Immortal Continuous Culture Cell Lines Originating from the Mammalian Ovary: The Chinese Hamster Ovary Cells

4.2.1 The Culture of Chinese Hamster Ovary Cells

Origin of Chinese Hamster Ovary Cells

- In 1955, ***Theodore T. Puck*** isolated epithelial cells from the ovaries of a Chinese Hamster (*Cricetulusgriseus*) (Puck and Marcus 1955).
- The cells were named **Chinese hamster ovary (CHO)** to designate their origin.
- During in vitro culture, some of the cells spontaneously immortalized, thus acquiring the capacity for unlimited division.
- CHO cells (CHO Pro3) are deficient in **proline production**. Thus, supplementation of proline is required during their in vitro culture.
- CHO Pro3 cells have mutated to create **CHO DG44**, a dihydrofolate reductase (**DHFR**)-deficient cell line.
- Since the original CHO cell line description, many variants have been developed for various purposes.
- A common CHO derivative is **CHO K1** (Xu X et al., 2011).
- **CHO K1** cells have lower DNA than the original CHO cells.
- A later mutation of CHO K1 generated CHO **BXB11** (also known as CHO **DUKX**), completely lacking DHFR activity.

NB: In humans, the DHFR gene is found in the q11 → q22 region of chromosome 5. DHFR has a critical role in regulating the tetrahydrofolate content of a cell, which is essential for purine and thymidylate synthesis, the precursors of nucleic acids. The synthesis of nucleic acids is a prerequisite for the proliferation and growth of cells Experimentally it has been shown that the mutant cells lacking DHFR require the amino acid glycine and thymidine to grow. DHFR is also involved in the tetrahydrobiopterin salvage from dihydrobiopterin.

Karyotype of Chinese Hamster Ovary Cells

- The stem line number of CHO cells is hypodiploid (chromosome frequency distribution 50 cells: $2n = 22$).
- It has been observed that CHO-DHFR cells on being treated with methotrexate hydrate exhibit chromosomal rearrangements arising from translocations and homologous recombination (Wurm and Hacker 2011).

Identification of Short Tandem Repeat Sequence
Currently, short tandem repeat sequences are used to identify the various mammalian cells, including cancer cells. The same may be applied to CHO cells also.

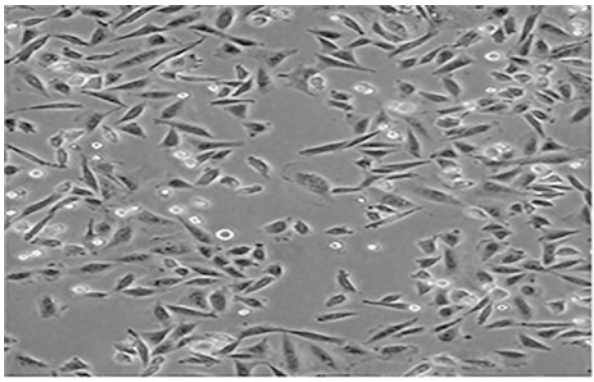

Fig. 3 Cultured Chinese hamster ovary (CHO) cells, an immortalized cell line as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/ccl-61)

Phenotype and Morphological Characteristics of Chinese Hamster Ovary Cells

- **Tissue type**
  Chinese hamster ovary.
- **Cell type**
  Epithelial.
- **Morphology**
  Epithelial (Fig. 3).
- **Shap**
  Round in suspension culture and flat in monolayer culture.
- **Diameter**
  (14–17) μm.

Marker Proteins Expression in Chinese Hamster Ovary Cells

- Do not express epidermal growth factor receptors (**EGFRs**).
- CHO-K1 cells lack dihydrofolate reductase (**DHFR**).
- The cystathionine γ-lyase enzyme involved in L-cysteine synthesis can be used as a metabolic selection marker in CHO cells.

Tumorigenicity of Chinese Hamster Ovary Cells

- In most of the studies, the CHO cells are recognized as noncancer cells. This is also supported by their **noncancer origin**.
- However, abnormal p53 function is observed in this cell line. Of note, in ~50–60% of all cancers, p53 mutation is observed.
- Based on p53 mutation and some more characteristics, this cell line is not considered a complete normal cell line.

Culture of Chinese Hamster Ovary Cells

1. **Suitable Culture Containers**
   T-75 flasks are preferred, but other vessels can also be used.
2. **Preparation of Cell Culture Medium**

F-12 medium, 10% FBS, 100 U/ml penicillin G, 100 μM streptomycin sulfate. Other media like RPMI-1640 could also be used.

NB: Use of antibiotics and antimycotics in culture medium is optional.

- The cell culture medium pH should be 7.2–7.4. Note that while human cell cytoplasm pH is 7.2, human blood pH is 7.4. Regular tracking of color changes in the culture medium is essential.
- Since metabolic products of cells (e.g., lactic acid) decrease the pH of the cell culture, medium sodium bicarbonate is used as a compensation additive.
- However, caution should be taken since a high concentration of sodium bicarbonate may alkalinize the cell culture medium.
- To diminish the action of accumulated metabolites from the actively growing cells, regular replacement of the cell culture medium is necessary.

Culture Process of Chinese Hamster Ovary Cells

- CHO cells can be cultured as adherent cells, forming a monolayer with a flat morphology.
- CHO cells also can be adapted to culture as suspension cells. Contrary to a monolayer, in suspension culture, more cells can be obtained using the same culture medium volume. Therefore, for recombinant protein production, CHO cells are usually well-adapted to suspension culture.

Chinese Hamster Ovary Cells as Adherent Cell Culture

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one CHO cell cryovial containing 1×10^6 cells/ml and aseptically transfer to the 75 mm flask.
- Incubate the flask in a CO_2 incubator with 8% CO_2 and 95% humidity at 37 °C.
- The following day, change the medium to remove DMSO and floating cells.
- Every 2 days, replace the old cell culture medium with the new medium.
- Within (2–3) days, (80–90) % of the 75 mm flask culture surface area will be covered and ready to be subcultured.
- At this time, the 75 mm flask will comprise nearly (10–12) million CHO cells.

NB: CHO cells are one of the fastest-growing cell lines.

- Some studies report that these cells grow better in **8% CO_2** rather than 5% CO_2.
- The CHO cells may form clusters during shaking or vigorous pipetting.

Chinese Hamster Ovary Cells as Suspension Cell Culture

- Allow the CHO cells to reach confluency (~2×10^5 cells/ cm^2) of almost 80–90%.
- Remove the cell culture medium.
- Rinse the cell with 1× PBS.

- Now detach the cells from the culture flask by adding warm (37 °C) trypsin (0.25 (w/v)%) and EDTA (0.53 mM).
- Observe the cells under an inverted microscope. Dispersal should happen within (3–5) min.
- In case detachment takes a longer time, the culture flask with added trypsin may be incubated at 37 °C for a few minutes.
- Additionally, one may need to mildly shake the culture vessel for cell detachment from the walls. However, there is a chance of cell clumping.
- Following detachment, the trypsin would be neutralized immediately with a 10 ml complete medium added to the flask. The used-up medium may be aseptically collected before trypsinization, which is devoid of dead cells, could be used for trypsin neutralization.
- To pellet down the cells, centrifuge for 5 min at 1000 rpm, 4 °C.
- Remove supernatant (trypsin/growth medium) from the tube.
- Decant the supernatant and resuspend the cells in a 5 ml medium.
- Remove an aliquot to determine viable cell count.
- Add **5×10^5 viable cells/ml in a sterile** spinner or shake flask.
- To maintain sufficient headspace for adequate gas exchange, add 100 ml medium in a 250 ml spinner flask or 75–100 ml medium) in a 250 ml shaker flask.
- Loosen the caps for gas exchange (if the flask caps do not contain a membrane filter) and place them at 37 °C in a humidified atmosphere of (5–10)% CO_2.
- In the next step for the Corning Spinners, set the impeller speed to 75–95 rpm. However, for the paddle-type impellers, speed may need to be decreased.
- Flasks can be rotated on an orbital shaker platform at 125–135 rpm.

NB: One can set the spinner flask impeller speed to (80–100) rpm. If the cell viability drops below 85%, reduce the speed by 10 rpm decrements until the viability is acceptable.

Once cell density reaches at least 1×10^6 cells/ml with a viability of at least 90% by day 3 post-planting for three passages, the cells may be considered well-adapted to suspension culture. Seeding density can then be reduced to (2×10^5–3×10^5) cells/ml. As mentioned above, a large-scale suspension culture of CHO cells is used for producing the various recombinant proteins.

Harvesting and Subculturing Chinese Hamster Ovary Cells

- Grow cells up to (70–80)% confluence.
- Rinse cells with 0.25 (w/v)% trypsin and 0.53 mM EDTA to remove the culture medium containing serum.
- Add 5 ml trypsin-EDTA to the culture flask and observe for cell layer detachment using an inverted microscope. This should occur within (3–5) min.
- This type of cell line succumbs to aggregation. Therefore, shaking the cell culture containers during trypsinization is not recommended.
- The trypsin-EDTA added cells may be put into a 37 °C incubator for a few minutes if the cells do not detach within (3–5) min.

- Following detachment, the trypsin will be neutralized immediately on supplementing 10 ml complete medium to the flask. The used-up medium may be aseptically collected before trypsinization if it does not contain dead cells and is used for trypsin neutralization.
- To pellet down the cells, centrifuge for 5 min at 1000 rpm, 4 °C.
- Remove supernatant (trypsin/growth medium) from the tube.
- Add 10 ml growth medium and gently aspirate cells with a pipette.
- Add cell suspension aliquots to culture vessels and **incubate at 37 °C in 8% CO_2**.
- For splitting, use 70–90% confluent culture in 1:4 to 1:8 proportion every 4–7 days.
- During subculturing, the medium needs to be changed as usual, that is, every 2–3 days.

Growth Characteristics of Chinese Hamster Ovary Cells

- **Culture properties**
  Either adherent or suspension.
- **Growth patter**
  Continuous growth (immortal cell line).
- **Population doubling time**
  14–17 h (rapid-growing).

Freezing and Thawing of Chinese Hamster Ovary Cells

Freeze CHO cells in complete growth medium (in suspended mode) with 10% DMSO at a concentration of (0.5–1) $\times$ 10^7cells/ml.

For adherent cells, perform freezing in a medium containing 10% DMSO with 1×10^6 cells/vial.

Usage of Chinese Hamster Ovary Cells

Various recombinant proteins with biomedical/therapeutic/industrial importance are produced using the CHO cell line. The level of recombinant protein production is on the scale of (3–10) grams per liter of culture.

- CHO cells are also a good model for radiation cytogenetics and tissue culture experiments.
- The study of the post-translational modifications of various recombinant human proteins can be done using the CHO cell line.
- Mutation of the epidermal growth factor receptors (EGFRs) is one of the reasons for the onset of various cancers, including breast and lung cancers. However, CHO cells do not express EGFR. **Therefore, this cell line can be used for screening the EGFR mutations and tumorigenic identification** (Kim JY et al. 2012).

Biosafety of Chinese Hamster Ovary Cells

Level 1.

4.3 The Culture of Continuous Cell Lines Originated from the Mammalian Prostate: LNCaP Cells and PC3 Cells

4.3.1 The Culture of LNCaP Prostate Cancer Cells

Origin of LNCaP Cells

In 1983, ***J.S. Horoszewicz*** *and colleagues* discovered LNCaP cells. For this discovery, a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male, with established metastatic prostate carcinoma, was utilized (Horoszewicz JS et al. 1983).

Karyotype of LNCaP Cells

- This is a **hypotetraploid** human cell line.
- The chromosome number ranges from (78 to 91), occurring in 22% of cells.
- Cells with chromosome counts of 86 (20%) or 87 (18%) occur at high frequencies.

Identification of Short Tandem Repeat Sequence

The use of short tandem repeat markers has been recommended for cell line authentication and identity confirmation of LNCaP cells.

Some prominent markers of LNCaP cells are mentioned below:

Amelogenin: X, Y
CSF1PO: 10, 11
D13S317: 10, 12
D16S539: 11
D5S818: 11, 12
D7S820: 9.1, 10.3
THO1: 9
TPOX: 8, 9
vWA: 16,18

Phenotypic and Morphological Characteristics of LNCaP Cells

- **Tissue type**
  Male prostate adenocarcinoma.
- **Cell type**
  Epithelial.
- **Morphology**
  Epithelial (Fig. 4).
- **Shape**
  Cuboidal.
- **Diameter**
  18 μm.

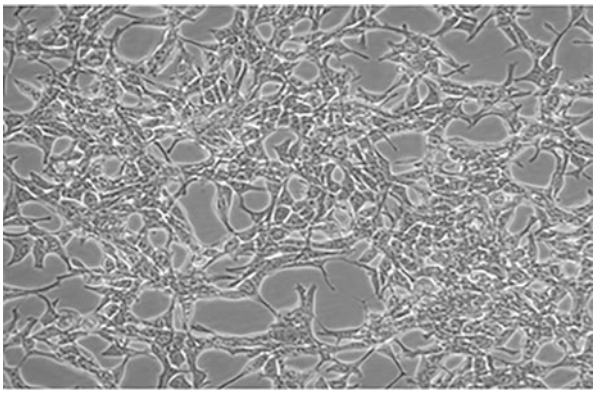

Fig. 4 Cultured LNCaP as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/crl-3313)

Marker Proteins Expression of LNCaP Cells

- Express androgen and estrogen receptors (Sampson N et al. 2013).
- Sensitive to androgen and estrogens.
- Express 5-alpha-reductase.
- Express acid phosphatase.
- Modulate 5-α-dihydrotestosterone and acid phosphatase production.
- Express prostate-specific antigen **(PSA)**.
- LNCaP cells are positive for CD9, CD10, CD19R, CD46, CD47, CD49F, CD54, CD58, CD59, CD99R, CD107, CD107a, CD107b, and CD147.

Tumorigenicity of LNCaP Cells

- Athymic nude mice develop tumors at the injection site.
- Male mice develop tumors earlier and at a greater frequency than females.
- Hormonal manipulations reveal a correlation of tumor development frequency with serum androgen levels (Horoszewicz JS et al. 1983).

Culture of LNCaP Cells

1. **Suitable Culture Containers**
   T-75 flasks are preferred though other culture vessels can be used.
2. **Preparation of Cell Culture Medium**
   RPMI-1640 contains 10% FBS, 100 U/ml penicillin G, and 100 μM streptomycin sulfate.

- ***NB: For healthy growth, (1–2) mM L-glutamine and 1 mM sodium pyruvate may be added to the culture medium.***
- ***The use of antibiotics or antimycotics in culture medium is optional.***
- ***While LNCaP cells are androgen receptor-positive and thus sensitive to androgens, adding androgens to the cell culture medium is generally not required unless necessitated by specific experimental conditions.***

- ***The cell culture medium pH should be within 7.2–7.4. Note that while human cell cytoplasm pH is 7.2, human blood pH is 7.4. Periodic inspection for color changes in the cell culture medium is essential.***
- ***Since metabolic products of cells (e.g., lactic acid) decreases the pH of the culture medium, supplementation with sodium bicarbonate is required.***
- ***However, caution should be taken since a high sodium bicarbonate concentration may alkalinize the culture medium.***
- ***To diminish the action of accumulated metabolites from the actively growing cells, regular replacement of the cell culture medium is required.***

3. **Culture Process of LNCaP Cell**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one LNCaP cryovial containing 8×10^5 cells and aseptically transfer to a 75 mm flask.
- Incubate the flask in a CO_2 incubator with 5% CO_2 and 95% moisture at 37 °C.
- The following day, change the medium to remove DMSO and floating cells.
- Every 2–3 days, replace the old cell culture medium with the new one.
- Within ~6 days, (80–90)% (75 mm) flask culture surface area will be covered, making it feasible for subculturing.

NB: High population density is required for proper LNCaP cell growth.

- Cells are highly adherent. Some reports suggest that for proper growth these cells require the culture flasks **to be coated with adhesive agents like poly-L-lysine or gelatin**.
- The monolayer of cells exhibits a high tendency to form a **cluster**. LNCaP cells are repeatedly pipetted up and down to dissociate the cluster before subculturing.

Harvesting and Subculturing LNCaP Cells

- Use a (90–95)% cell monolayer T-75 flask, which should contain **$6–8 \times 10^5$ cells/sq. cm**.
- Remove the cell culture medium.
- Rinse the cells with PBS to remove residual cell culture medium and serum.
- This cell line tends to aggregate. Therefore, shaking the cell culture containers during trypsinization is not recommended.
- The trypsin-EDTA-supplemented cells may be put into a 37 °C incubator for a few minutes if they do not detach within (3–5) min.
- Following detachment, the trypsin is promptly neutralized using 10 ml complete medium supplementation. The used-up medium may be aseptically collected before trypsinization if this medium does not contain dead cells and is used for trypsin neutralization.
- To pellet down the cells centrifuge for 5 min at 1000 rpm at 4 °C.
- Remove the supernatant (trypsin/growth medium) from the tube.

- As usual, incubate the cells at 37 °C in 5% CO_2 and 95% moisture.
- Every 6–7 days, use the trypsin-EDTA to split the cells in **1:2 to 1:6 proportions**.
- Make sure to renew the medium every 2–3 days.
- Add 10 ml growth medium and gently aspirate cells with a pipette.
- Take new culture vessels and add sufficient cell suspension.

Growth Characteristics of LNCaP Cells

- **Culture properties**
  Adherent/loosely adherent in culture.
  High tendency to form clusters.
- **Growth pattern**
  Continuous culture (immortal metastatic cancer cell line).
- **Population doubling time**
  Relatively slow-growing prostate cancer cell line in general, doubles in (48–60) h.

Freezing and Thawing of LNCaP Cells

Same as discussed for other cells.

Usage of LNCaP Cells

- *LNCaP cells are useful* for oncology research, specifically for prostatic adenocarcinoma study.
- To understand the effects of androgens/steroids on prostate cancer development.
- Androgen-insensitive LNCaP cells have been developed and are widely used to understand the development and complexity of non-androgen-sensitive prostate cancer.

Biosafety of LNCaP Cells

Level 1.

4.3.2 The Culture of PC3 Prostate Cancer Cells

Origin of PC3 Cells

- In 1979, the PC3 cell line was established from **metastatic grade IV prostate cancer** in a 62-year-old Caucasian male.
- PC3 cells are a poorly differentiated adenocarcinoma cell line (Kaighn ME et al. 1979; Tai S et al. 2011).

Karyotype of PC3 Cells

- The PC3 cell line is near-triploid with 58–62 modal chromosomes.
- In general, 20 marker chromosomes are found in PC3 cells.
- This cell line is devoid of normal N2, N3, N4, N5, N12, and N15 markers.
- The Q-band analysis revealed an absence of Y chromosomes (Ohnuki Y et al. 1980; Chen 1993; van Bokhoven A et al. 2001).

Identification of Short Tandem Repeat Sequence

Currently, short tandem repeat sequences are used to identify the various mammalian cells including cancer cells. The same may be applied to PC3 cells also.

Some prominent markers are as follows:

Amelogenin:
CSF1PO: 11
D13S317: 11
D16S539: 11
D5S818: 13
D: 8, 1
THO1: 6, 7
TPOX: 8, 9
vWA: 17

Phenotype and Morphological Characteristics of PC3 Cells

- **Tissue type**
  Human male prostate.
- **Cell type**
  Epithelial cells.
- **Morphology**
  Epithelial (Fig. 5).
- **Shape**
  Cuboidal or spindle.
- **Diameter**
  65–24 μm.

NB: This cell line is more characteristic of neuroendocrine or small cell carcinoma than adenocarcinoma.

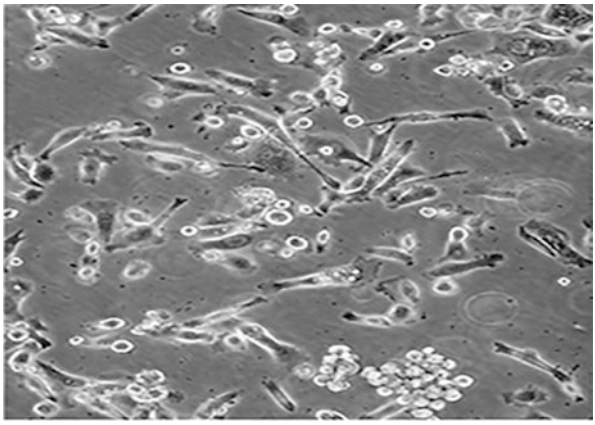

Fig. 5 Cultured PC3 cells as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/crl-1435)

Marker Proteins Expression in PC3 Cells

- PC3 cells are positive for CD9, CD46, CD47, CD99R, CD107a, CD107b, CD147, CD49b, CD49f, CD55, CD59, CD99R, CD104, CD44, and CD97.
- Lack testosterone-5-α-reductase.
- Lack of acid phosphatase.
- Lack of prostate-specific antigen **(PSA**).
- Lack of prostate-specific membrane antigen.
- Do not respond to fibroblast growth factors, glucocorticoids, and androgens.
- PC3 cells express aberrant p53 with a C deletion in codon 138, developing a nonsense codon at locus 169 (on a loss of heterozygosity), and are PTEN deficient.
- PC3 cells exhibit high TGFα and EGFR expression.

Tumorigenicity of PC3 Cells

- These cells produce a tumor in athymic nude mice.
- PC3 cells exhibit the characteristics of **invasive and highly metastatic cancer cells.**

Culture of PC3 Cells

1. **Suitable Culture Containers**
   T-75 flasks are preferred, but other culture vessels could also be used.
2. **Preparation of Cell Culture Medium for PC3 Cells**
   F-12 K medium containing 10% FBS, 100 U/ml penicillin G, and 100 μM streptomycin sulfate.

NB: RPMI-1640 can be used instead of an F-12 K medium.

- ***For healthy growth, 1–2 mM L-glutamine and 1 mM sodium pyruvate may be added to the culture medium.***
- ***Serum concentration may be increased to 15% if the growth rate remains low even after the addition of L-glutamate and sodium pyruvate.***
- ***The use of antibiotics or antimycotics in the culture medium is optional.***
- ***The culture medium pH should be 7.2–7.4. Note that while human cell cytoplasm pH is 7.2, human blood pH is 7.4. Regular monitoring of culture medium color changes is essential.***
- ***Since metabolic products of cells (e.g., lactic acid) decrease the pH of the culture medium, sodium bicarbonate supplementation is used. However, caution should be taken as a too high sodium bicarbonate concentration may alkalinize the medium.***
- ***To diminish the action of accumulated metabolites on the actively growing cells, regular replacement of the cell culture medium is necessary.***

3. **Culture Process of PC3 Cell**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one PC3 cryovial containing 1×10^6 cells/ml and aseptically transfer to a 75 mm flask.
- Incubate the flask with 5% CO_2 and 95% humidity at 37 °C.
- The following day, change the medium to remove DMSO and floating cells.
- Replace the old cell culture medium with the new medium every 2 days.
- Within **5–6 days, 80–90%** of the 75 mm culture flask, the surface area will be covered and ready for subculture.
- At this time, the 75 mm flask should contain ~80,00,000 PC3 cells.

NB: 75 mm flask is also called a T-75 flask.

Harvesting and Subculturing of PC3 Cells

- Remove and discard culture medium.
- Briefly rinse the cell layer with 0.25% (w/v) trypsin and 0.53 mM EDTA or PBS to remove all serum traces containing trypsin inhibitor.
- Add 5 ml trypsin-EDTA solution to the flask and observe under an inverted microscope until the cell layer is dispersed.
- Within 2–4 min, the cells should be detached.
- Add 10 ml complete growth medium and gently aspirate cells by pipetting.
- Centrifuge the culture at low speed.
- Remove the supernatant.
- Make a suspension of the cells in the complete cell culture medium.
- Split the medium in a **1:3 to 1:6** proportion.
- Incubate the cultures at 37 °C in a CO_2 incubator.

Growth Characteristics of PC3 Cells

- **Culture properties**
  Naturally, these cells are adherent in nature. They form a cluster in soft agar. However, experimental models revealed the capacity for significant adaptation in suspension culture.
- **Growth pattern**
  Continuous growth (immortal cell line).
- **Population doubling time**
  24–33 h if freshly subcultured.

Freezing and Thawing of PC3 Cells

PC3 cells can grow either as adherent or in suspension. The separate freezing and thawing procedures for adherent and suspension growth patterns are as detailed below.

Freezing of Adherent PC3 Cells

Normal standard procedure is used to freeze the PC3 cells.

- Before freezing, screen cells for bacterial, yeast, or fungal contamination.
- Trypsinize cells (standard protocol).
- Resuspend the cells in a cell culture medium to neutralize the trypsin-EDTA, and centrifuge them at 1000 rpm, 4 °C for 3–5 min.
- Remove the supernatant and add DMSO containing cell freezing medium to the cell pellet.
- Make a cell suspension and put 1 ml cell suspension per cryovial.
- Now, use the standard slow freezing procedure to free the cells in liquid nitrogen.

Thawing of Adherent PC3 Cells

- Use the general fast thawing procedure for the cells. Briefly, the procedure is as follows:
- Warm up a water bath and allow the temperature to reach 37 °C.
- Take a cryovial from the liquid nitrogen and immediately put it into the water bath.
- The following thawing put the vial content in a 9 ml cell culture medium in a Petri plate.
- Incubate the culture plate at 37 °C in a CO_2 incubator.
- Change the cell culture medium the following day or as soon as the cells are attached.

Freezing of Nonadherent PC3 Cells

- Before freezing, screen the cells for bacterial, yeast, or fungal contamination.
- Take a 50 ml Falcon tube and aseptically transfer the cell suspension.
- Centrifuge the tube at 1000 g for 15 min at 4 °C.
- Make fresh freezing medium containing 90% FBS or complete cell culture medium and 10% mammalian cell culture-grade DMSO.
- Label cryogenic vials with date, cell type, and user's initials.
- Following centrifugation, remove the supernatant and add a freezing medium to the cell culture pellet. Now make a cell suspension.
- Put 1 ml cell suspension into 1.5 ml cryovial and use a standard slow freezing procedure.

Thawing of Adherent PC3 Cells

- The thawing procedure is the same for adherent and nonadherent cells. The thawing of nonadherent cells has already been discussed above.
- Pipette the vial contents (~1 ml) into a T25 flask.
- Slowly add 4 ml cold culture medium at a rate of nearly 1 drop every 10 s, with occasional swirling. Add another 5 ml culture medium.
- Place the flask in an incubator.
- Since the freezing medium contains dimethylsulfoxide (DMSO), spin down cells after (6–12) h and resuspend in the fresh, prewarmed medium in a new T25 flask.

Usage of PC3 Cells

- PC3 cells have high metastatic potential compared to that of moderate extent for DU145 cells as well as LNCaP cells, which have a low metastatic potential.
- PC3 cells are useful for investigating biochemical changes in advanced prostate cancer cells with the capacity for high-level invasion and metastasis and for assessing responses to chemotherapeutic agents.
- PC3 cells can be used to create subcutaneous tumor xenografts in mice to investigate the tumor microenvironment and therapeutic drug effects.
- Suitable for the study of neuroendocrine adenocarcinoma.
- Suitable for the study of androgen nonresponsive prostate cancer.
- Table 2 distinguishes the culturing attributes of LNCaP and PC3 cell lines, originating from the human prostate.

Biosafety of PC3 Cells

Level 1.

4.4 The Culture of Continuous Cell Lines Originated from the Mammalian Cervix: HeLa Cells

4.4.1 Culture of HeLa Cells

Origin of HeLa Cells

- In 1952, Dr. ***George Gey*** isolated a cell from the cervical cancer tissue of a 31-year-old Afro-American woman named ***Henrieta Lacks*** (Gey GO et al. 1952; Jones HW Jr. et al. 1971).
- ***The nomenclature of HeLa originated from the initials of her first (He) and last (La) names.***

Table 2 Comparative description of LNCaP and PC3 cells

LNCaP cells	PC3 cells
Cells share common features with adenocarcinoma	Cells are characteristics of neuroendocrine carcinoma (SCNC)
Express androgen and estrogen receptors	Do not express androgen or estrogen receptors
Sensitive to anti-androgen and anti-estrogen therapy	Not sensitive to anti-androgen and anti-estrogen therapy
Express prostate-specific antigen (PSA)	Do not express prostate-specific antigen (PSA)
Express 5-alpha reductase	Do not express 5-alpha reductase
These cells are positive for CD9, CD10, CD19R, CD46, CD47, CD49F, CD54, CD58, CD59, CD99R, CD107, CD107a, CD107b, and CD147	These cells are positive for CD9, CD46, CD47, CD99R, CD107a, CD107b, CD147, CD49b, CD49f, CD55, CD59, CD99R, CD104, CD44, and CD97
Express acid phosphatase	Do not express acid phosphatase
Grow as clusters	Do not grow as clusters
Low-level invasion and metastatic potential	High-level invasion and metastatic potential

- ***HeLa is the first human cell line that survived* in vitro *culture for indefinite generations.***

Karyotype of HeLa Cells

- Over time, HeLa cells exhibit cytogenetic instability, and at present, it has several strains.
- Most HeLa cells have 70–90 chromosomes with over 20 translocations, some of which are highly complex involving multiple chromosomal rearrangements.
- Four (M1–M4) typical HeLa chromosome markers are established with one Ml, one M2, five M3, and two M4 copies, as revealed by G-banding patterns.
- M1 is a rearranged long arm. It includes the centromere of chromosome 1 and the long arm of chromosome 3.
- M2 is a combination of chromosome 3 short arm and chromosome 5 long arm.
- M3 is an isochromosome of the chromosome 5 short arm.
- M4 consists of chromosome 11 long arm and an arm of chromosome 19 (Macville M et al. 1999).

Identification of Short Tandem Repeat Sequence

Currently, short tandem repeat sequences are used to identify the various mammalian cells including cancer cells. The same may be applied to HeLa cells also.

Some prominent markers are as follows:

Amelogenin: X
CSF1PO: 9, 10
D13S317: 12, 13.3
D16S539: 9, 10
D5S818: 11, 12
D7S820: 8, 12
THO1: 7
TPOX: 8, 12
vWA: 16,18

Phenotype and Morphological Characteristics of HeLa Cells

- **Tissue type**
  Human cervical.
- **Cell type**
  Epithelial.
- **Morphology**
  Epithelial (Fig. 6).
- **Shape**
  The usual appearance is a spreading cell, ranging from a sphere to that of a "**fried egg.**" This progressed to filopodia radial extension in between, which the cytoplasm is subsequently filled with.
- **Diameter**
  20 μm.

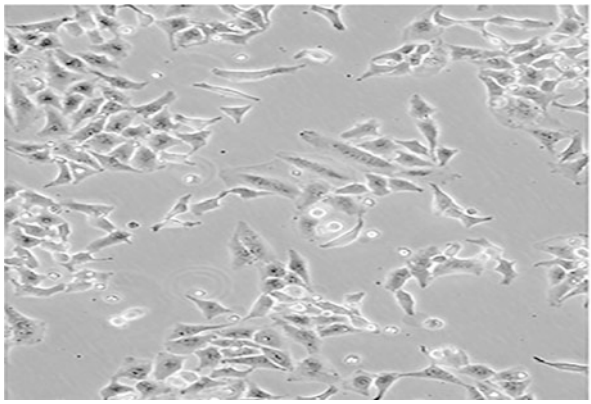

Fig. 6 Cultured HeLa, cervical cancer cells as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/ccl-2)

Marker Proteins Expression in HeLa Cells

The following are the marker proteins expressed by HeLa cells:

- Keratin
- CD51
- α-tubulin
- Vimentin
- Thermin

Tumorigenicity of HeLa Cells

HeLa cells are tumorigenic in athymic nude mice.

Culture of HeLa Cells

1. **Suitable Culture Containers**
   T-75 flasks are preferred, although other culture vessels can also be used.
2. **Preparation of Cell Culture Medium for HeLa Cells**
   DMEM with high glucose (4.5–5 g/L glucose), supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

NB: ATCC recommended Eagle's Minimal Essential Medium (EMEM) instead of DMEM for HeLa cell culture.

- For rapid growth, 1–2 mM L-glutamine and 1 mM sodium pyruvate may be added to the cell culture medium. Serum concentration may be increased to 15% if growth rates remain slow after L-glutamate and sodium pyruvate addition.
- The use of antibiotics and antimycotics in the culture medium is optional.
- The culture medium pH should be within (7.2–7.4). Note that while human cell cytoplasm pH is 7.2, human blood pH is 7.4. Regular checking of medium color changes is essential.

- Since metabolic products of cells (e.g., lactic acid) decrease the pH of the cell culture medium, treatment with sodium bicarbonate is essential. However, caution should be taken since a high sodium bicarbonate concentration may alkalinize the cell culture medium.
- To diminish the effects of accumulated metabolites, regular replacement of the cell culture medium is necessary.

3. **Culture Process of HeLa Cells**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one HeLa cryovial containing 1×10^6 cells/ml and aseptically transfer to a 75 mm flask.
- Incubate the flask in a CO_2 incubator with 4–5% CO_2 and 95% moisture at 37 °C.
- The following day, change the medium to remove DMSO and floating cells.
- HeLa cells are one of the fastest-growing.
- Culture containers are usually confluent within 2–3 days.
- Generally, there is no need for medium change before harvesting the cells.

Harvesting and Subculturing HeLa Cells

- Use a (90–95)% cell monolayer T-75 flask.
- Remove the cell culture medium.
- Rinse the cells with PBS to remove residual cell culture medium and serum.
- This cell line shows a tendency to aggregate. Therefore, shaking the cell culture containers during trypsinization is not recommended.
- The trypsin-EDTA added cells may be put into a 37 °C incubator for a few minutes if the cells do not detach within 3–5 min.
- Following detachment, the trypsin will be neutralized immediately with 10 ml complete medium to the flask. The used-up medium may be aseptically collected before trypsinization if this medium does not contain dead cells and is used for neutralization of trypsin.
- To pellet down the cells, centrifuge for 5 min at 1000 rpm and 4 °C.
- Remove supernatant (trypsin/growth medium) from the tube.
- As usual, incubate the cells at 37 °C in 5% CO_2 and 95% moisture.
- Every 6–7 days, use trypsin-EDTA to split the cells in **1:2 to 1:6** proportions.
- Make sure to renew the medium every 2–3 days.
- Add 10 ml growth medium and gently aspirate cells with a pipette.
- Take new culture vessels and sufficient cell suspension, and split at a ratio of **1: 5–1:10.**
- Incubate cultures at 37 °C, 5% CO_2, and 95% moisture.

Growth Characteristics of HeLa Cells

- **Culture properties**
  Adherent.
- **Growth pattern**

Continuous growth (immortal cell line). The cells become immortal or cancerous due to the integration of the genome with the human papillomavirus (**HPV**) 18.

In a startling discovery in 2013, it was shown that the scrambled HPV genome was inserted near the **c-Myc** proto-oncogene in Henrietta Lack's genome. This insertion of the HPV genome resulted in its constitutive expression and the rapid replication of HeLa cells.

- **Population doubling time**

  The doubling time of HeLa cells is just **24 h**. This indicates that HeLa cells are one of the fastest-growing mammalian cancer cells in the world.

  HeLa cells grow so fast that if they contaminate other mammalian cell cultures, the other cell's culture growth is rapidly superseded.

Freezing and Thawing of HeLa Cells

Freezing of HeLa Cells

- Prepare and sterilize filter freezing media, containing 10% DMSO, and 20% FBS in DMEM (~1 ml/cryovial). Bring cell medium, PBS, and trypsin to room temperature.
- Aspirate the cell medium.
- The freezing procedure is the same as discussed for other cells.

Thawing of HeLa Cells

The thawing procedure is the same as discussed for other cells.

Usage of HeLa Cells

- HeLa cells are the first immortal cell lines ever recognized. Since this cell line can be passaged as many times as possible, it created a new dimension for cell culture-based assay systems.
- **In 1952,** the HeLa cells were used for the first time by ***Jonas Salk*** to develop the world's first polio vaccine.
- **In 1953,** HeLa cells were mistakenly mixed with a liquid that caused their chromosomes to unclamp. Unclamping of chromosomes leads to clear visibility of the total chromosome numbers (**46**) of HeLa cells. This provided a crucial breakthrough to identify abnormalities.
- **In 1965,** HeLa cells were used by ***Henry Harris*** and ***John Watkins*** for human genome mapping.
- **In 1984,** virologist ***Haraldzur Hausen*** tested a sample from Lacks's original biopsy and detected the human papillomavirus (**HPV) 18**. Using HeLa cells, he discovered that HPV 18 causes **cervical cancer**, paving the way for a preventive vaccine.
- **In 2014, the** engineers and chemists at *Penn State University* implanted **synthetic nanomotors** into HeLa cells for the first time. The technology may 1 day enable doctors to destroy cancer cells inside the body.
- The use of HeLa cells helped in the inception of virology.

- Over the years, scientists infected HeLa cells with various viruses including **HIV, herpes, measles, and mumps.** This led to the discovery of **CD4**, the surface protein encoded by T-cell, which is used by HIV to enter inside a cell. HeLa cells infected with HIV allowed for testing for anti-HIV drugs. Researchers also learned that the measles virus constantly mutates when it infects HeLa cells, making it harder to eradicate the disease.
- ***More recently, microbiologists found that the Zika virus cannot multiply in HeLa cells.***

Biosafety of HeLa Cells

Level 2. Cells contain the **human papilloma virus-18 (HPV-18).**

4.5 The Culture of Continuous Cell Lines Originated from the Mammalian Lung: BEAS-2B Cells and A549 Cells

4.5.1 The Culture of BEAS-2B Lung Cancer Cells

Origin of BEAS-2B Cells

- The normal human bronchial epithelial cells of a healthy individual were isolated and transformed through **Simian virus 40 (SV40) large T-antigen**. This experimental transformation leads to the creation of BEAS-2B cells.
- Thus, this cell line is extensively used to understand the basics of malignant transformation.
- Further transformation of this cell line is possible through in vitro experiments via **RAS, CYP2A13**, and **SPR1 overexpression**.
- Exposure to chromium, arsenates, cadmium, cigarette smoke, and uranium also resulted in deleterious effects on this cell line (Yang ZH et al. 2002; Zhang T et al. 2012, 2014; Zhao and Klimecki 2015).

Karyotype

Not specified.

Identification of Short Tandem Repeat Sequence

Currently, short tandem repeat sequences are used to identify the various mammalian cells including cancer cells. The same may be applied to BEAS-2B cells also. These methods are currently being used to identify human cell lines.

Some prominent markers to screen BEAS-2B cells are as follows:

Amelogenin: XY
CSF1PO: 9, 12
D13S317: 1
D16S539: 12
D5S818: 12, 13
D7S820: 10, 13

THO1: 7, 9.3
TPOX: 6, 11
vWA: 17, 18

Phenotype and Morphological Characteristics of BEAS-2B Cells

- **Tissue type**
  Human bronchus of the lung.
- **Cell type**
  Virus transformed squamous epithelial cells (Fig. 7).
- **Morphology**
  Prior studies demonstrate that the BEAS-2B (cells) phenotype can be influenced by the presence or absence of fetal bovine serum (**FBS**) in the culture medium (Lechner and LaVeck 1985; Miyashita M et al. 1989).

Exposure of BEAS-2B cells to FBS is associated with the following changes:

- Differentiation of squamous cells
- Cytokine secretion alteration
- Altered response to toxicants

Marker Protein Expression in BEAS-2B Cells

- Express keratin.
- Express SV40 T antigen.

Tumorigenicity of BEAS-2B Cells

- These cells can create tumors in athymic nude mice.
- The **BEAS-2B cells** can be employed to screen the heavy metal-induced transformation and carcinogenesis in cell models via distinct experimental models.

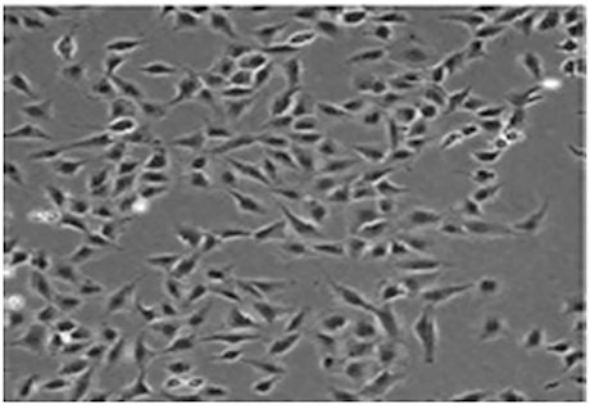

Fig. 7 Cultured BEAS-2B cells, transformed human lung cells as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/crl-9609)

Culture of BEAS-2B Cells

1. **Suitable Culture Containers**

   T-75 flasks are preferred, but other culture vessels may also be used.

2. **Preparation of Cell Culture Medium**

   BEGM, also known as LHC-9 with modification or BEBM Bullet Kit (available from Clonetics), is the best culture medium.

NB: As mentioned above, the addition of FBS to any BEAS-2B culture medium (RPMI/DMEM,* etc.*) produces a significantly altered phenotype and therefore should be avoided. The addition of antibiotics or antimycotics is optional.

3. **Culture Process of BEAS-2B Cells**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one BEAS-2B cell cryovial containing **1×10^6 cells/ml** and aseptically transfer to a 75 mm flask.
- Incubate the flask in a CO_2 incubator equipped with 3.5% CO_2 and 95% moisture at 37 °C.
- The following day, change the medium to remove DMSO and floating cells.
- The BEAS-2B cells are one of the fastest-growing cell lines.
- Culture containers may reach confluence within 4–5 days.
- Change the culture medium every 2–3 days.

NB: Cells are grown at 37 °C in a 3.5% CO_2 (these cells seem to grow best on Costar plastic) environment.

- If the cells have been frozen in a serum medium, they should be washed several times to remove the serum before being put into culture vessels.
- Reports describe the use of BEAS-2B cells in varied culture conditions.
- Several reports precoated the culture vessels for culturing BEAS-2B cells. A mixture of coating agents such as 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I, and 0.01 mg/ml bovine serum albumin is dissolved in BEBM.

Harvesting and Subculturing of BEAS-2B Cells

- The cell harvesting procedure is the same as previously discussed for other cells.
- Resuspend the pellets in a complete cell culture medium and split at a ratio of **1: 2–1:6.**
- Incubate cultures at 37 °C.

NB: The BEAS-2B cells are sensitive to over-trypsinization.

- Cells may form clumps if shaken too much.
- These cells should be **subcultured before reaching confluence** since confluent cultures rapidly undergo terminal squamous differentiation.

Growth Characteristics of BEAS-2B Cells

- **Culture properties**
  Adherent.
- **Growth pattern**
  Continuous growth (immortal cell line).
- **Population doubling time**
  26 h.

Freezing and Thawing of BEAS-2B Cells

In general, the freezing cell density should be **1–5 × 10^6 cells/ml**. Freezing and thawing procedures are as usual for other cells.

Usage of BEAS-2B Cells

- BEAS-2B cells are an ideal in vitro experimental model of pulmonary epithelium.

Some of the experimental circumstances where this cell line could be useful are as follows:

- For in vitro toxicity evaluation of various environmental pollutants including cigarette smoke.
- To understand acute lung injury (**ALI**), acute respiratory distress syndrome (**ARDS**), and the role of various **surfactant proteins** in wound healing.
- To study the neoplastic transformation of the pulmonary epithelial cells. This property can be used for screening chemical and biological agents inducing or affecting differentiation and/or carcinogenesis.
- To study pneumococcal infection mechanisms (Garcia-Canton C et al. 2013).

Biosafety of BEAS-2B Cells

Biosafety **level 2** is recommended.

4.5.2 The Culture of A549 Lung Cancer Cells

Origin of A549 Cells

- The A549 cell line was established in 1972 by ***Giard and colleagues*** (Giard DJ et al. 1973).
- A549 cells originated from an explant culture of lung cancer (**alveolar squamous epithelial cells**) from a 58-year-old Caucasian male.
- This human alveolar basal epithelial cell line has been used as a **type II pulmonary epithelial cell model** (Lieber M et al. 1976; Foster KA et al. 1998).

Karyotype of A549 Cells

The modal chromosome number of this cell line is 66 (44% cases), making it a hypotriploid cell line.

- Cells with 64 (22%), 65, and 67 chromosomes also occur at relatively high frequencies; however, the frequency of higher ploidies is low (0.4%).
- The most important markers present in all cells are as follows: der(6)t(1;6) (q11; q27); del(6) (p23); del(11) (q21), del(2) (q11), M4, and M5.
  Approximately 60% of the cells contain two X and two Y chromosomes.
  In ~40% of cells, both the Y chromosome may be lost.
- Chromosomes N2 and N6 have single copies in cells while N12 and N17 usually have four copies.

NB: Cytogenetic information is based on initial seed stock at ATCC.

Identification of Short Tandem Repeat Sequence

The use of short tandem repeat markers has been recommended for cell line authentication and identity confirmation of A549 cells.

Some characteristic short-term repeat sequences of A549 cells are as follows:

Amelogenin: X, Y
CSF1PO: 10, 12
D13S317: 11
D16S539: 11, 12
D5S818: 11
D7S820: 8, 11
THO1: 8, 9.3
TPOX: 8, 11
vWA: 14

Phenotype and Morphological Characteristics of A549 Cells

- **Tissue type**
  Alveoli of the human lung.
- **Cell type**
  Type II alveolar epithelial cells.
- **Morphology**
  Epithelial-like (Fig. 8).
- **Shape**
  If cultured in vitro, these cells grow as monolayers, adherent to culture flasks.
- **Diameter**
  The diameter of A549 cells as estimated using inverted microscopy and TEM images is 14.93 and 10.59 μm, respectively.

Marker Proteins Expression in A549 Cells

These cells are positive for the following marker proteins:

Keratin
Lecithin
Surfactant protein C, an alveolar epithelial type II (ATII) marker.

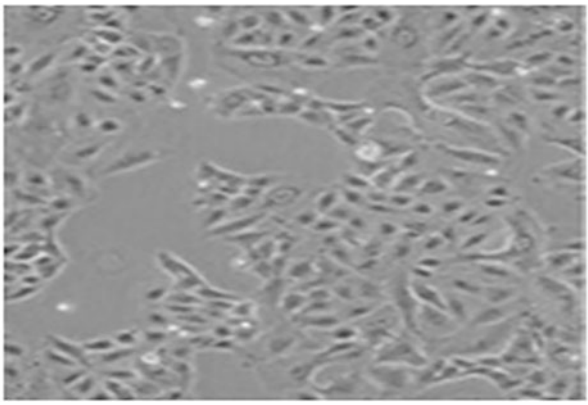

Fig. 8 Cultured (A549) human lung cancer cells as observed through an inverted microscope. (Image adapted from ATCC database: https://www.atcc.org/products/ccl-185)

Thyroid transcription factor-1 (*TTF-1*
Aquaporin-5, an (ATI) marker

Tumorigenicity of A549 Cells

Subcutaneous or intravenous injection of A549 cells generates tumors in athymic nude mice.

Culture of A549 Cells

1. **Suitable Culture Containers**
   T-75 flasks are preferred, although other vessels can also be used.
2. **Preparation of Cell Culture Medium**
   Cells grow well in F-12 K medium [with 10% FBS and penicillin (100 U/ml), streptomycin 100 μg/ml].

NB: In general, cells grow very well without any additives.

Cells also grow well in DMEM with high glucose ((4.5–5) gm glucose/liter of medium).

3. **Culture process of A549 Cells**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one A549 cell cryovial containing ~1 × 10^6 cells/ml and aseptically transfer to a 75 mm flask.
- Incubate the flask in a CO_2 incubator equipped with 5% CO_2 and 95% moisture at 37 °C.
- The following day, change the medium to remove DMSO and floating cells.
- Within (2–4) days, cells reach the confluence with (6 × 10^3 to 6 × 10^4) cell/cm^2.

- Change the medium every 2–3 days if the flask does not reach confluency within that time.
- Cells are now ready to be subcultured.

NB: Some reports suggest that before starting any experiment A549 cells have to be passaged at least three times after thawing.

Furthermore, a maximum of 20 passages (after thawing) must not be exceeded due to the possibility of further transformation.

Harvesting and Subculturing of A549 Cells

- Take a 70–75% confluent T-75 flask containing around (**6×10^3 to 6×10^4**) cells/cm^2.
- Remove and discard the culture medium.
- The harvesting procedure is the same as discussed previously for other cells.
- Do low-speed centrifugation and remove the supernatant.
- Resuspend the pellets with a complete cell culture medium and split at a **1:2 to 1:4 ratio**.
- Incubate the cultures at 37 °C in a 5% CO_2 and 95% moisture environment.

Growth Characteristics of A549 Cells

- **Culture properties**
  Grow adherently as a monolayer with an epithelial morphology and tightly compacted small cells.
- **Growth pattern**
  Continuous growth (immortal cell line).
- **Population doubling time**
  Around 22 h.

Freezing and Thawing of A549 Cells

Freezing of A549 Cells

The process begins with the preparation of a fresh stock solution that is subsequently precooled on ice for at least 1 h before use. It is prepared as a 2× concentrated stock and is diluted with cell suspension.

Composition of Stock Solution

The freezing medium is prepared using a 60% complete cell culture medium, 20% FCS, and 20% DMSO. The final concentration after **1:1 mixing** of cell suspension and freezing medium is adjusted to comprise 80% complete cell culture medium (containing cells), 10% FCS, and 10% DMSO. The entire freezing process is summarized as follows:

- Mix cell suspension (**2×10^6 cells/ml**) in 1:1 ratio with precooled freezing medium (e.g., 10 ml cell suspension with 10 ml freezing medium). This results in a final cell concentration of **1×10^6 cells/ml.**

- Place the cells in a cryovial and keep them at 0 °C for half an hour and then at -20 °C for 2 h.
- Place the cryovial in a freezing container before keeping it inside a freezer (−70 °C to −80 °C) for 24 h.
- This leads to a freezing rate of approximately 1 °C per minute.
- After 24 h, place the frozen cryovial into liquid nitrogen for long-term storage.

Thawing of A549 Cells

- The procedure for thawing the A549 cell line is the same as previously discussed for other cells.
- Check confluency after **24 h**. Cells should be subcultured after attaining 70% confluency. Less confluent cultures should be supplemented with 20 ml fresh prewarmed complete cell culture medium per T-75 flask, followed by growth to reach approximately 70% confluency.
- Passage of the cultured cells at least three times before use.

Usage of A549 Cells

- These cells grow as a monolayer adherent culture and could be used for transformation experiments.
- These cells serve as models of alveolar type II pulmonary epithelium and are useful for examining the metabolic processing of lung tissue and possible mechanisms of lung-specific drug delivery.
- A549 cells are also used for viral research and associated with diverse protein expressions as a consequence of viral infection and tuberculosis pathogenesis.
- This cell line efficiently forms tumors in nude mice xenograft models.
- A549 cells are a useful model for anticancer drug testing (Lieber M et al. 1976).
- Table 3 distinguishes the culturing traits of BEAS-2B and A549 lung cancer cell lines for their implicit experimental screening.

Biosafety of A549 Cells

Level 1.

4.6 The Culture of Continuous Cell Line Originated from the Mammalian Kidney: HEK-293 Cells and VERO Cells

4.6.1 The Culture of HEK-293 Kidney Cancer Cells

Origin of HEK-293 Cells

- In 1973, Dr. ***Alex Van der Eb's*** laboratory from the *University of Leiden* generated a cell line from **human embryonic kidney (HEK)** cells of an aborted normal (not suffering from any disease) human fetus by the transformation with sheared adenovirus 5 DNA.
- In this transformation, a **4.5-kbp viral DNA** was incorporated into **chromosome 19** of the HEK cells.

Table 3 Differences between BEAS-2B and A549 lung cancer cell lines

BEAS-2B cells	A549 cells
Simian virus 40 (SV40) large T-antigen immortalized human lung bronchial epithelial cell line	Originated from an explant culture of lung adenocarcinoma tissue (alveolar squamous epithelial cells) from a 58-year-old Caucasian male Characterized as type II alveolar epithelial cells
Cause tumors in athymic or nude mice	Cause tumors in athymic or nude mice
As marker proteins express keratin and SV-40 T antigen	As marker proteins express keratin, lecithin, surfactant protein C, and thyroid transcription factor 1 and aquaporin-5
Produces low levels of reactive oxygen species (ROS)	Produces high levels of reactive oxygen species (ROS)
Cell phenotype can be influenced by the presence or absence of fetal bovine serum (FBS) in the culture medium Exposure of BEAS-2B to FBS is associated with squamous differentiation, alterations in cytokine secretion, and response to toxicants	No differentiation is noticed in the presence of FBS

- Thus, HEK terminology originated from human embryonic kidney cells and 293 is an arbitrary number or the number of experiments in *Alex Van der Eb's* laboratory.
- A mutant version of the HEK-293 cells having mutant SV40 antigen has been isolated and characterized and named **HEK-293-T cells** (Alex van der Eb 2012).

Karyotype of HEK-293 Cells

- This is a hypotriploid human cell line.
- The modal chromosome number is 64.
- Chromosomal abnormalities include three copies of the X chromosome and four copies of the 17th and 22nd chromosomes.
- The presence of multiple X chromosomes and the lack of any Y chromosome-derived sequence suggested the fetal source to be a female.

Identification of Short Tandem Repeat Sequence

The use of short tandem repeat markers has been recommended for cell line authentication and identity confirmation of HEK-293 cells.

Some prominent characteristic short tandem repeat sequences are as follows:

Amelogenin: X
CSF1PO: 11, 12
D13S317: 12, 14
D16S539: 9, 13
D5S818: 8, 9
D7S820: 11, 1
THO1: 7, 9.3.

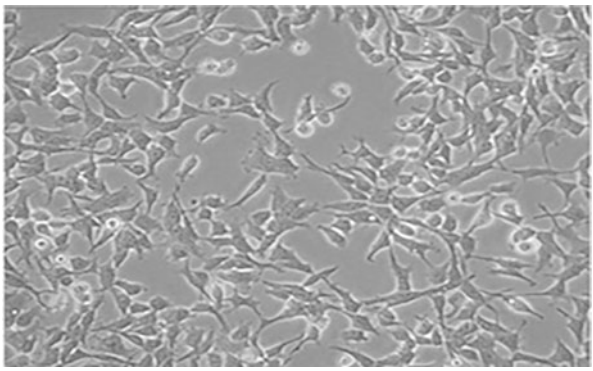

Fig. 9 An inverted microscopic view of cultured HEK-293 cells, a transformed cell line from the human kidney. (Image adapted from ATCC database; https://www.atcc.org/products/crl-3249)

TPOX: 11
vWA: 16,19

Phenotype and Morphological Characteristics of HEK-293 Cells

- **Tissue type**
  Human embryonic kidney.
- **Cell type**
  More closely resembles adrenal cells rather than typical kidney epithelial cells (Fig. 9).
- **Morphology**
  Epithelial.
- **Shape**
  Round.
  Cells have large nuclei, occupying approximately two-thirds of cytoplasmic volume.
- **Diameter**
  ~(15–20) μm.

Marker Proteins Expression in HEK-293 Cells

Various proteins are expressed by this cell line. The prominent marker proteins are vitronectin, Nestin, CD133, Notch1, and nerve growth factor receptor. The other proteins expressed by the HEK-293 cell line are Snai2, Sox9, Sox10, Phox2b, and Ascl1.

Tumorigenicity of HEK-293 Cells

- Passage level-dependent tumorigenicity, with 100% expression when the passage number exceeds 65.
- No tumor is induced for less than 52 passage numbers.
- These results are the findings of studies on nude mice models.

Culture of HEK-293 Cells

1. **Suitable Culture Containers**

   T-75 flasks are preferred, although other cell culture vessels can also be used.

2. **Preparation of Cell Culture Medium**

   Eagle's Minimum Essential Medium (EMEM, ATCC formulated) with 10% FBS **(**Liste-Calleja L et al. 2013).

NB: In general, the cells do not need any additives for healthy growth. Cells also grow well in DMEM with high glucose (4.5–5 g/L).

3. **Culture Process of HEK-293 Cells**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one HEK-293 cryovial containing **1×10^6 cells/ml** and aseptically transfer to the 75 mm flask.
- Incubate the flask in a 5% CO_2 and 95% moisture environment at 37 °C.
- The following day, change the medium to remove DMSO and floating cells.
- This is a very rapid-growing cell line.
- Renew the medium (**2–3**) times a week, keeping the cell count between **1×10^5 to 3×10^5** cells/ml.
- Do not allow cell concentration to exceed 1×10^6 cells/ml.
- The approximate cell number to attain 100% confluency for this cell line in a T-75 flask is 1×10^7 cells.
- Figure 10 summarizes the chronological steps for culturing HEK-293 cells in a T-75 flask.

Harvesting and Subculturing of HEK-293 Cells

- Cells will reach the splitting number of between **1×10^5 and 3×10^5 cells/ml** in ~2–3 days.
- HEK-293 cells can easily be detached by washing out with a growth medium.
- There is no need to trypsinize. Just take the 10 ml pipette and wash them out by pipetting up and down with the cell culture medium.
- Collect and do low-speed centrifugation.
- Aspirate the supernatant, resuspend with cell culture medium, and split at a **1: 4–1:8 ratio**.

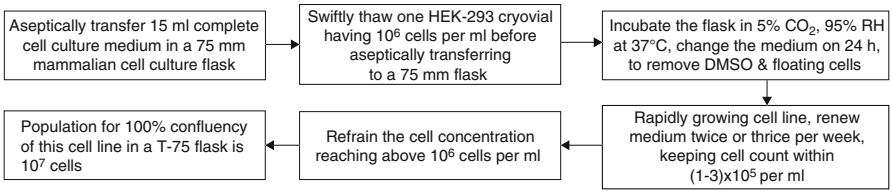

Fig. 10 Chronological steps in HEK-293 cell culturing in a T-75 mammalian culture flask

Growth Characteristics of HEK-293 Cells

- **Culture properties**
  Loosely adherent cells, but can also grow well as a cell suspension. The cells can be removed while being inside the culture flask without adding trypsin-EDTA.
- **Growth pattern**
  Continuous growth (immortal cell line).
- **Population doubling time**
  (20–24) h. Doubling time depends upon the medium composition.

Freezing and Thawing of HEK-293 Cells

Cells can be stored as stock in liquid nitrogen at **(2–5)** $\times$ $\mathbf{10^6}$ **cells/ml** in a growth medium containing **5–10%** DMSO. Use the standard procedure for freezing and thawing.

Usage of HEK-293 Cells

- Growth and maintenance of this cell line are easy with high reproducibility as compared to others. Thus, this cell line is preferable to other less robust and slow-growing cell lines.
- HEK293 is one of the easiest cell lines to transfect with various vectors including adenovirus. This cell line can be used for the proliferation of adenovirus vectors. Of note, while the cell line contains several adenovirus genes, it lacks the hazardous E1 and E2 genes.
- These cells are commonly used for protein expression and the production of recombinant retroviruses. For example, a recombinant activated protein C was synthesized, isolated, and later tested as a novel protein for the sepsis treatment.
- This cell line is being used to examine the interactions between different proteins and drugs. For example, the effects of drugs on sodium channels were examined by using this cell line.
- Around the year 2005, a HEK293 cell line variant, called **HEK 293 T,** was developed. In this cell line, **SV40 Large T antigen** is present. Of note, the transfected SV40-containing plasmids can undergo episomal replication, enhancing the concentration of recombinant protein/retroviral production (Thomas and Smart 2005).

Biosafety of HEK-293 Cells

Level 2 (cells contain adenovirus).

4.7 The Culture of VERO Kidney Cancer Cells

4.7.1 Origin of VERO Cells

The Vero cell line was isolated from the kidney of a normal adult African green monkey on March 27, 1962, by ***Y. Yasumura*** and ***Y. Kawakita*** at the Chiba University in Chiba, Japan (Yasumura and Kawakita 1963).

NB: African green monkey belongs to the Chlorocebus species, formerly called Cercopithecus aethiops; this group of monkeys has been split into several species.

4.7.2 Karyotype of VERO Cells

- Detailed karyotype analysis of VERO cells is now available.
- In ~66% of the cells, the modal chromosome number is 58, so chromosomes are recognized as hypodiploid.
- Regarding the various chromosomes, the following data is available: Absence of A3, A4, B4, and B5 chromosomes while B2, B3, and B7 are occasionally paired, B9, C1, and C5 are mostly paired.
- The rest of the chromosomes are mostly present as a single copy.
- ~1.7% of cells exhibit higher ploidies.
- Over 50% of the chromosomes in most of the cells belong to structurally altered marker chromosomes.

4.7.3 Identification of Short Tandem Repeat Sequence

The scrutiny of short tandem repeat markers is recommended for cell line authentication. These methods are currently being used to identify human cell lines. The method can also be used for identifying the monkey-derived cells including the VERO cell line.

4.7.4 Phenotype and Morphological Characteristics of VERO Cells

- **Tissue type**
  African green monkey kidney.
- **Cell type**
  Epithelial.
- **Morphology**
  Retain epithelial morphology (Fig.11).

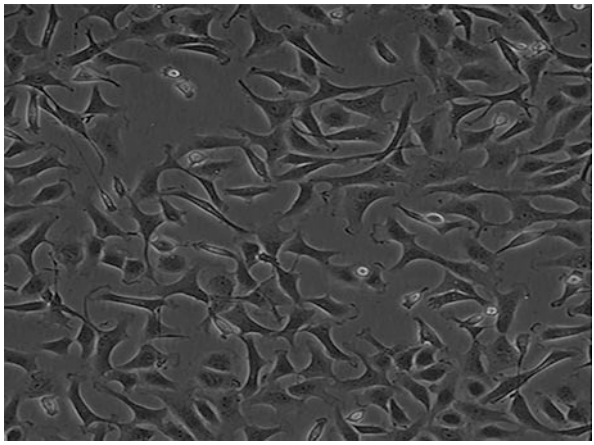

Fig. 11 An inverted microscope view of cultured VERO cell line (African green monkey kidney cancer cells). (Image adapted from ATCC database; https://www.atcc.org/products/ccl-81.5)

- **Diameter**
  ~17 μm.

4.7.5 Marker Proteins Expression in VERO Cells

- Do not secrete interferon-alpha or beta, although they do express interferon-alpha and beta receptors.
- Do not express cyclin-dependent kinase inhibitors, CDKN2A and CDKN2B.

4.7.6 Tumorigenicity of VERO Cells

Develop tumors, particularly at long passage numbers in athymic nude mice.

4.7.7 Culture of VERO Cells

1. **Suitable Culture Container**
   T-75 flasks are preferred, although other culture vessels can also be used.
2. **Preparation of Cell Culture Medium**
   EMEM (ATCC formulated) with 10% FBS and antibiotics and antimycotics (optional).

NB: DMEM medium with high glucose (4.5–5 g/L) content can also be used as an alternative to EM5EM. Generally, the cells do not need additives for healthy growth.

3. **Culture Process of VERO Cells**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one VERO cell cryovial and aseptically transfer to a 75 mm flask.
- Incubate the flask in an incubator equipped with 5% CO_2, and 95% humidity, at 37 °C.
- The following day, change the medium to remove DMSO and floating cells.
- This is one of the fastest-growing cell lines.
- Renew medium **2–3** times a week (Ammerman NC et al. 2008; M. C. de O. Souza et al. 2005).

4.7.8 Harvesting and Subculturing of VERO Cells

- Take a 70–75% confluent T-75 flask.
- Remove and discard the culture medium.
- The harvesting procedure is the same as discussed previously for other cells.
- Perform low-speed centrifugation and remove the supernatant.
- Resuspend the pellets with a complete cell culture medium and split at a **1:2 to 1: 4 ratio**.
- Incubate cultures at 37 °C in a 5% CO_2 and 95% moisture, equipped environment.

4.7.9 Growth Characteristics of VERO Cells

- **Culture properties**
  Adherent in nature.
- **Growth pattern**
  Continuous growth (immortal cell line).
- **Population doubling time**
  18 h.

4.7.10 Freezing and Thawing of VERO Cells

As detailed for other cell lines.

4.7.11 Usage of VERO Cells

The Vero cell line is one of the most common and well-established mammalian cell lines involved in assessing the effects of chemicals, toxins, and other substances at the molecular level.

Some important uses of VERO cells are as follows:

- The toxins originated from ***Escherichia coli,*** tested on VERO cells, and called VERO toxins.
- The toxin originated from ***Shigella dysenteriae*** **and** is called **Shiga toxins and** is tested on this cell line.
- VERO cells are also utilized for culturing various animal viruses and to maintain the virus stocks for research purposes.
- Also utilized to check the effects of various pharmaceutical agents/drugs/virus plaque-forming unit (**PFU**).
- These cells are also useful for monitoring the infection by other viruses such as ***influenza A, influenza B,*** and ***adenoviruses*** (Govorkova EA et al. 1996; Hasler and Wigand 1978).
- As host cells for eukaryotic parasites, especially ***trypanosomatids***.
- Table 4 distinguishes the HEK-293 and VERO kidney cell lines for their explicit experimental identification.

4.7.12 Biosafety of VERO Cells

Level 1

4.8 The Culture of Continuous Cell Lines Originated from the Mammalian LIVER: HEPG2 Cells

4.8.1 The Culture of HepG2 Liver Cancer Cells

Origin of HepG2 Cells

This is a well-differentiated human hepatocellular carcinoma cell line that originated from a 15-year-old African-American boy.

Table 4 Differences between HEK-293 and VERO kidney cell lines

HEK-293 cells	VERO cells
The HEK-293 cells originated by transforming a normal embryonic kidney cell from an aborted fetus with adenovirus 5 DNA Believed to be originated from a **neural crest** adjacent to the kidney	The Vero cell line was isolated from the kidney of a normal adult African green monkey
This is a hypotriploid human cell line with a modal chromosome number of 64	This is a hypodiploid African green monkey cell line with a modal chromosome number of 58, which occurs in 66% of cells
Diameter: (15–20) microns	Diameter: 17 microns
Retains epithelial morphology; however, the shape is spherical with a big nucleus engaging two-thirds of cell volume	Retains epithelial morphology
These cells express vitronectin, nestin, CD133, Notch1, nerve growth factor receptor, Snail2, Sox9, Sox10, Phox2B, and Asc11	VERO cells express various virus receptor proteins, and cell adhesion molecules, including claudins, various integrins, CD9, epidermal growth factor receptors, and prohibitin These cells do not express CDKN2A and CDKN2B The cells do not secrete interferon-α or β
Doubling time: ~(20–24) h	Doubling time: 18 h
Culture medium: EMEM (ATCC formulated) with 10% FBS or DMEM with high glucose	Culture medium: EMEM (ATCC formulated) with 10% FBS, or DMEM with high glucose
Loosely adherent cells	Adherent cells
Usefulness: transfection with viruses, and recombinant mammalian protein production	Usefulness: screening of toxins, chemicals, and drugs

Karyotype of HepG2 Cells

- The modal chromosome number is 55.
- HepG2 cells exhibit a hyperdiploid karyotype: 52(47–54) < 2n > XY, +2, +14, +17, +20, +2mar, t(1;21) (p22.2;p11–12), i(17q)/der(17)t(17;17)(p11;q11).

Identification of Short Tandem Repeat Sequence

The use of short tandem repeat markers has been recommended for cell line authentication and identity confirmation of HepG2 cells.

Some specific short tandem repeat sequences are as follows:

Amelogenin: X, Y
CSF1PO: 10, 11
D13S317: 9, 13
D16S539: 12, 13
D5S818: 11, 1
D7S820: 10
THO1: 9
TPOX: 8, 9
vWA: 17

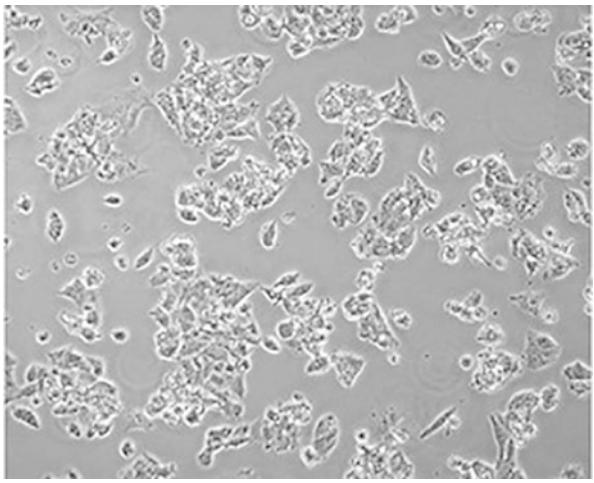

Fig. 12 Cultured HepG2cells, transformed human liver cancer cells as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/hb-8065)

Phenotype and Morphological Characteristics of HepG2 Cells

- **Tissue type**
  Carcinoma affected the human liver.
- **Cell type**
  Epithelial.
- **Morphology**
  Retain epithelial cell-like morphology (Fig. 12).
- **Shape**
  Round.
- **Diameter**
  10 μm.

Marker Proteins Expression in HepG2 Cells

- Hepatitis B virus surface antigens have not been yet detected.
- The cells express 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase.
- Figure 13a depicts the chemical structure of 3-hydroxy-3-methylglutaryl-CoA reductase, having numerous H^+ donating sites (terminal –OH, NH_2 functional groups). Such H^+-donating abilities decipher a chemical reduction catalyzing ability of 3-hydroxy-3-methylglutaryl-CoA reductase.
- The cells demonstrate decreased APO-A1 (apolipoprotein A1) mRNA expression.
- The cells exhibit increased catalase mRNA expression in response to gramoxone, a fast-acting, nonselective, contact herbicide that induces oxidative stress (Fig. 13b).

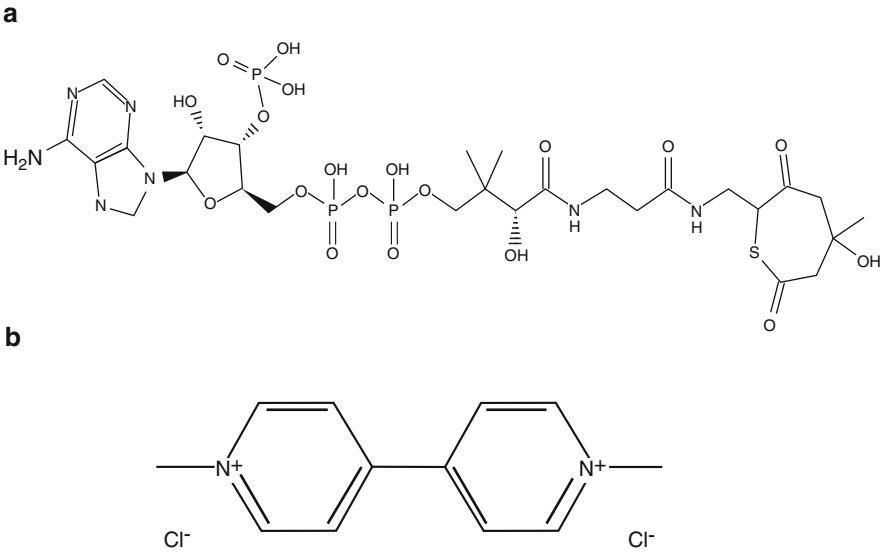

Fig. 13 (**a**) Chemical structure of 3-hydroxy-3-methylglutaryl-CoA reductase. (**b**) Chemical structure of gramoxone, the Cl- being instrumental in accumulating oxidative stress

Tumorigenicity of HepG2 Cells

HEPG2 cells are tumorigenic in athymic nude mice. However, some reports suggest their nontumorigenic background in nude mice.

Culture of HepG2 Cells

1. **Suitable Culture Containers**
   T-75 flasks are preferred, but other culture vessels can also be used.
2. **Preparation of Cell Culture Medium**
   EMEM was supplemented with high glucose (4.5–5 g/l), low $NaHCO_3$ (1.5 g/l), and 1 mM L-glutamine supplemented with 10% FBS.

NB: DMEM and RPMI1640 can also work well.

3. **Culture Process of HepG2 Cells**

- Take a 75 mm mammalian cell culture flask.
- Aseptically transfer 15 ml complete cell culture medium.
- Rapidly thaw one HepG2 cell cryovial.
- Incubate the flask in an incubator equipped with 5% CO_2 and 95% humidity at 37 °C.
- Change the medium on the following day to remove DMSO and floating cells.
- Cells are relatively slow-growing, taking (6–7) days to reach 80–90% confluency.
- Figure 14 briefs the above steps for an easy follow-up for the readers.

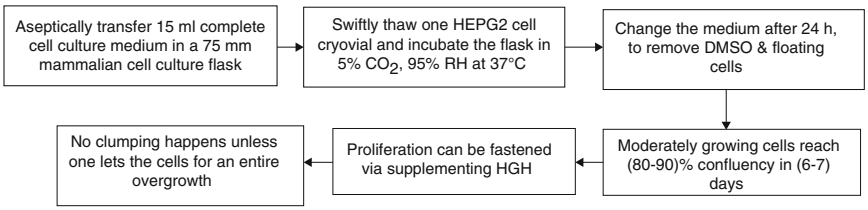

Fig. 14 Chronological steps in the culture process of HepG2 cells

NB. Although the growth rate is relatively slow, cells are highly metabolically active. Need cell culture medium replacement every 2–3 days.

- Proliferation can be stimulated by supplementing human growth hormone (HGH).
- Cells start as islands.
- To make a single-cell suspension of HepG2 cells at every passage, flush them 3–4 times through a 21G needle.
- No cell clumping happens unless one lets them completely overgrow.

Harvesting and Subculturing of HepG2 Cells

- Take a (70–75)% confluent T-75 flask.
- Remove and discard the culture medium.
- The harvesting procedure is the same as discussed previously for other cells.
- Do low-speed centrifugation and remove the supernatant.
- Resuspend the pellets with a complete cell culture medium and split at a **1:2 to 1: 4 proportion**.
- Incubate the cultures at 37 °C, 5% CO_2, and 95% moisture (Donato MT et al. 2015).

NB: Cells tend to clump and form aggregates. Resuspension before seeding needs to be vigorous since on being kept around for some time, the cells tend to clump in suspension.

Growth Characteristics of HEPG2 Cells

- **Culture properties**
  Epithelial-like cells.
  Show adherent properties.
  Grow as a monolayer with small aggregates.
- **Growth pattern**
  Continuous (immortal cell line).
- **Population doubling time**
  48 h.

Freezing and Thawing of HEPG2 Cells

As detailed for other cells.

Usage of HEPG2 Cells

- HEPG2 cells are used for large-scale production of multiple proteins, including transferrin, fibrinogen, plasminogen, albumin α-fetoprotein, α_2-macroglobulin, α1-antitrypsin, α1-antichymotrypsin, haptoglobin, ceruloplasmin, plasminogen, complement-C4, C3 activator, α1-acid glycoprotein, α2-HS glycoprotein, β-lipoprotein, and retinol-binding protein.
- HepG2 cells are also used as a model system for studying liver metabolism and xenobiotic toxicity.
- This cell line is also used in the detection of environmental, dietary cytotoxic, and genotoxic agents (i.e., cytoprotective, anti-genotoxic, and co-genotoxic moieties).

Biosafety of HEPG2 Cells

Level 1.

4.9 The Culture of Continuous Cell Lines Originated from the Mammalian Blood: JURKAT Cells and RAW Cells

4.9.1 The Culture of JURKAT Blood Cancer Cells

Origin of JURKAT Cells

The Jurkat cell line was established from the peripheral blood of a 14-year-old boy suffering from acute T cell leukemia by ***Schneider and colleagues*** and was originally designated as JM.

Karyotype of JURKAT Cells

- This is a pseudodiploid human cell line.
- The modal chromosome number is 46, with 74% occurring frequency and 5.3% polyploidy.
- The karyotype is 46, XY,-2,-18, del (2) (p21p23), del (18) (p11.2).
- Both normal X and Y chromosomes are present in most of the cells.
- Several mutations were noticed in the genome of this cell line (Louis Gioia, A et al. 2018).

Identification of Short Tandem Repeat Sequence

The use of short tandem repeat markers has been recommended for cell line authentication and identity confirmation of Jurkat cells. These methods are currently being used to identify human cell lines.

Some characteristic short tandem repeat sequences are as follows:

Amelogenin: X, Y
CSF1PO: 11, 12
D13S317: 8, 12

D16S539: 11
D5S818: 9
D7S820: 8, 12
THO1: 6, 9. 3
TPOX: 8, 10
vWA: 18

Phenotype and Morphological Characteristics of JURKAT Cells

- **Tissue type**
  Peripheral blood.
- **Cell typ**
  T-lymphocytes.
- **Morphology**
  Lymphoblastic.
- **Shape**
  Round (Fig. 15).
- **Diameter**
  (11.5 ± 1.5) μm.

Marker Proteins Expression in JURKAT Cells

- Jurkat cells express **CD3** on their surface, similar to normal T cell CD3 expression.
- These cells express cell surface **TCR** similar to normal T cells.
- These cells secrete **IL-2,** similar to normal T cells.

Tumorigenicity of JURKAT Cells

In mice, abnormal expression of the exosomal valosin-containing protein (**VCP**) is exhibited. VCP is a membrane ATPase involved in ER homeostasis and ubiquitination.

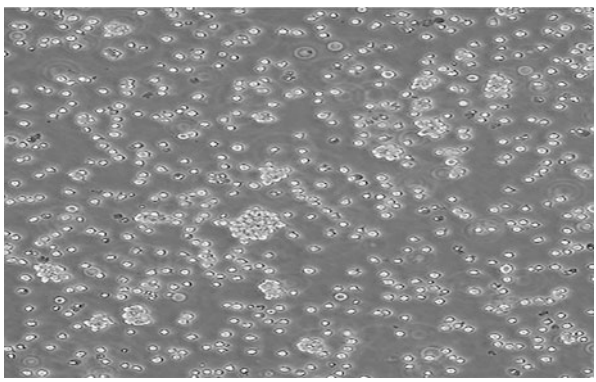

Fig. 15 Cultured JURKAT cells, the human T-cell cancers as observed through an inverted microscope. (Image adapted from ATCC database, https://www.atcc.org/products/tib-152)

Culture of JURKAT Cells

1. **Suitable Culture Containers**

   T-25 flasks are preferable, but other culture vessels can also be used.

2. **Preparation of Cell Culture Medium**

   RPMI-1640 with high glucose (4.5–5 g/L) containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 1.5 gm/L sodium bicarbonate. 50 μM β-mercaptoethanol (also known as 2-mercaptoethanol) must be added to the cell culture medium for the proper growth of JURKAT cells.

NB: 2-Mercaptoethanol is a reducing agent required to prevent ROS from affecting mouse cells. It is generally not necessary for normal human cells.

3. **Culture Process of JURKAT Cells**

- Thaw a cryovial as soon as possible containing **1×10^6 cells**.
- Immediately after thawing transfer the cells into a 15 ml conical tube containing a 9 ml medium.
- Low-speed centrifugation at 4 °C to pellet down the cells.
- Discard the supernatant and very gently resuspend the cells in 10 ml warm medium.
- Now, divide the cell content (5 ml each) into two **T-25 flasks**.
- Replace the medium every 2 days.
- When cells are **$(6–8) \times 10^5$ cells/ml**, split them at 1:4 in a fresh medium. Add 10 ml to a 25 ml culture flask.
- While cells can reach **3×10^6 cells/ml** density corresponding to 100% confluency, it is therefore advised not to grow cells at more than a maximum of **1×10^6 cells/ml**.

NB: JURKAT cells show a tendency to clump. However, the clumps can be easily dispersed by pipetting up and down.

Maintenance of JURKAT Culture

- This is a suspension culture.
- So, the cell culture is maintained either by the addition of a fresh medium or complete replacement of the cell culture medium following centrifugation.
- For resuspension, the medium should be added to such an extent so that cell concentration reaches **1×10^5** viable cells/ml.
- Culture the cells and maintain the cell concentration between (**10^5 to 10^6**) viable cells/ml.
- Every **2–3 days** interval (or depending upon the number of cells), replace the old medium with the fresh one.

Growth Characteristics of JURKAT Cells

- **Culture properties**

  Suspension type.

- **Growth pattern**
  Continuous (immortal cell line).
- **Population doubling time**
  48 h.

Freezing and Thawing of JURKAT Cells

- Make a freezing medium by adding 5% mammalian cell culture-grade DMSO and 95% complete cell culture medium ((v/v).
- Add 1 ml freezing medium per **5×10^6 cells.**
- Use standard slow freezing procedure as discussed for other cells.

Usage of JURKAT Cells

- To produce interleukin-2 (**IL-2**).
- For understanding T cell signaling, this cell line may be utilized.
- This cell line is also utilized to understand various chemokine receptors susceptible to viral entry, particularly **HIV**.
- To study T-cell leukemia.
- To study drug and radiation susceptibility of cancers.
- Jurkat and its variants are also used to monitor protein expression, viral interactions, and cancer biochemistry.

Biosafety of JURKAT Cells

Level 1.

4.9.2 The Culture of RAW 264.7 Blood Cancer Cells

Origin of RAW Cells

- RAW 264.7 cell line originated by the transformation of macrophages isolated from BALB/C mice by the **Abelson leukemia virus.**
- The cell line was developed by ***WC Raschke*** in 1978 (Raschke WC et al. 1978).

Karyotype of RAW Cells

Not specified.

Identification of Short Tandem Repeat Sequence

Screening of short tandem repeat markers is recommended for cell line authentication and identity confirmation of RAW 264.7 cells. These methods are currently being used to identify human cell lines. A similar method can also be utilized for mice cells.

Phenotype and Morphological Characteristics of RAW Cells

- **Tissue type.**
  This is an **Abelson murine leukemia virus-induced tumor (ascites).**
- **Cell type**
  Transformed macrophage cell line.

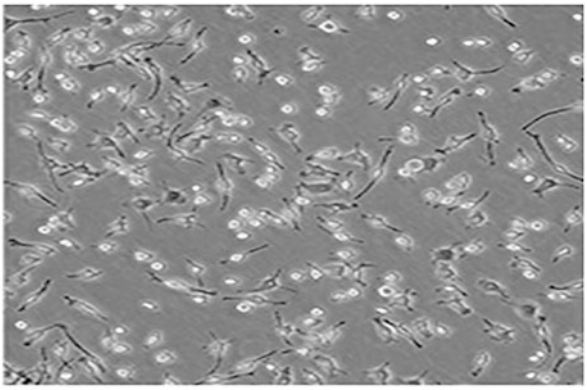

Fig. 16 Cultured RAW cells, the transformed mouse macrophage cancer cells as observed through an inverted microscope. (Image adapted from ATCC database; https://www.atcc.org/products/tib-71)

- **Morphology**
  Macrophage (**M1**), can transdifferentiate to (**M2**).

NB: This phenotype is sensitive to bacterial lipopolysaccharide (LPS).

- **Shape**
  Round to fried **egg-like (**Fig. 16**)**.
- **Diameter**
  30 μm.

Marker Proteins Expression in RAW Cells

- CD14, CD11b, F4/80.
- **Receptor expression:** complement (C3).
- Antigen expression: H-2d.

Tumorigenicity of RAW Cells

Not exactly known.

Culture of RAW Cells

1. **Suitable Culture Containers**
   T-25 flasks are preferred, but other culture vessels can also be used.
2. **Preparation of Cell Culture Medium**
   The complete medium consists of **DMEM**, high glucose (4.5–5 g/ L), 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate.
3. **Culture Process of RAW Cells**

- Take two T-25 flasks.
- Take a cryogenic vial containing RAW cells (~2.5×10^6 in number).

- After quick thawing, put the cells in a 10 ml cell culture medium and do low-speed centrifugation.
- Remove the supernatant containing DMSO.
- Add 20 ml, complete cell culture medium to the pellet.
- Do a pipetting up and down for cell dispersion.
- Divide the cells into two T-25 flasks (10 ml each).
- Incubate in a CO_2 incubator.
- The flask will be around (70–75)% confluent (the confluency needed for sub-culturing) in (2–3) days.

NB: The mature cells may be loosely adherent and tend to float. The adherent or nonadherent growth profile of RAW cells is dependent on trypsin activity. In general, studies have used trypsin to detach the growing RAW cells from the basal layer. RAW 264.7 adheres to tissue culture-grade plastic through cation-dependent integrin receptors and other cation-independent receptors, predominantly the murine scavenger receptors (MSRs) **(Fraser I et al.** 1993**).**

Harvesting and Subculturing of RAW Cells

- Aspirate the growth medium by tilting the flask to allow the medium collection at a corner.
- The cells are mildly adherent. However, the cell line is sensitive to trypsinization.
- Therefore, mildly use a cell lifter or scaper to dislodge the cells from the culture containers.
- Flash the flask with the fresh complete cell culture medium.
- Collect the cell suspension from the flask.
- Split the cells at 1:3, 1:4, or 1:5 dilutions (all are acceptable).
- Monitor for growth and refresh media when necessary.

NB: According to ATCC, the RAW cells may change phenotype after 18 passages. Thus, more than 18 passages are not recommended during the culturing.

Under specific conditions, RAW cells may be trans-differentiated into osteoclasts. Thus, cultural conditions must be properly maintained (Taciak B et al. 2018).

Growth Characteristics of RAW Cells

- **Culture properties**

  Flattened adherent/ semi-adherent growth with monolayer cells. RAW 264.7 cells adhere to tissue culture-grade plastic through cation-dependent integrin receptors and other cation-independent receptors, predominantly the **MSRs**.

  To reduce adhesion during routine culture, the RAW 264.7 cells can be grown on sterile non-tissue-grade plastic (ultra-dish Petri dishes). Adherence of macrophages to these plates is mediated by αMβ2 (**CR3**) integrins, and this interaction can be readily reversed using a cation chelator such as EDTA. This adhesive interaction is also sufficiently weak so that the cells can be detached by the sheer force of the medium flowing over the cells.

- **Growth pattern**
  Continuous (immortal cell line).
- **Population doubling time**
  (12–14) h.

NB: Fast-growing mammalian cells.

Freezing and Thawing of Raw Cells

Freezing of Raw Cells

Considering the nature of these cells, it is recommended that freezer stocks be made as soon as a new vial of low passage number cells is cultured.

- Grow to a density of **1×10^6 cells** in a 20 ml culture medium. This can be done in a larger 175 cm^2 flask.
- Centrifuge at 1000 rpm for 5 min to pellet cells.
- Resuspend in 3.6 ml FCS/FBS and add 400 µl DMSO in a drop-wise manner.
- Pipette 1 ml into a cryovial.
- Quickly place on ice for 10 min and then at -20 °C for 2 h. Finally, store at −80 °C overnight.
- The following day, transfer to liquid nitrogen or keep under −80 °C for up to 6 months.

Thawing of RAW Cells

Use standard cell thawing procedure as already discussed for other cells.

Usage of RAW Cells

- These cells represent a **standard mouse model of the macrophage cell line**.
- As a cell line, RAW cells are easy to work with and available in large numbers, facilitating certain experiments that generally require a large amount of starting material (e.g., biochemical studies).
- It is easy to propagate this cell line.
- This cell line harbors a high efficiency for DNA transfection and is sensitive to RNA interference (Hartley JW et al. 2008).
- This cell line is utilized to understand the host immune response against microbial infections.
- RAW cells support the replication of murine noroviruses.
- RAW cells produce lysozymes.
- As both JURKAT cells and RAW cells arise from mammalian blood, it is important to have a thorough acquaintance of their fundamental distinctions from an experimental viewpoint. Table 5 contains such basic distinctions, which could prove handy to a beginner in mammalian cell culture learner.

Table 5 Differences between JURKAT and RAW blood cells

JURKAT cells	RAW cells
These cells are immortalized human T lymphocyte cells	These cells are macrophage-like, Abelson leukemia virus-transformed cells derived from BALB/c mice
Morphology: lymphoblast	**Morphology:** macrophage (M1), with the capacity to differentiate to M2
Diameter: (11.5–12.5) microns	**Diameter:** 30 microns
Shape: round shape	**Shape:** round to fried egg
Population doubling time: 48 h	**Population doubling time:** 12–14 h Fast-growing mammalian cells
Marker proteins: CD3, CD4, TCR, etc.	**Marker proteins:** CD14, CD11B, F4/80, C3 complement receptors, H-2d antigen
Secretion: High-level IL-2, but no lysozyme synthesis	**Secretion:** Low-level IL-2, as well as produces lysozymes
Medium to culture: RPMI-1640 with high glucose (4.5–5 gm/liter), containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 1.5 gm/L sodium bicarbonate and 50 micro molar beta-mercaptoethanol	**Medium to culture:** Dulbecco's Modified Eagle's medium (DMEM), with high glucose (4.5–5 gm/liter), 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1.5 gm/L sodium bicarbonate
Usefulness: these cells are mainly used to study acute T cell leukemia, T cell signaling, and the expression of various chemokine receptors susceptible to viral entry, particularly HIV	**Usefulness:** transfection studies, cellular responses to microbes and their products, propagation of the virus, RNA interference study, etc.

***NB: Disadvantages: Like many cell lines, macrophages exhibit significant heterogeneity* in vivo*. Given this heterogeneity, it can be difficult to extrapolate results obtained using RAW cells to* in vivo *macrophage functioning. This may be an important factor to consider when judging the experimental suitability of RAW cells*.**

Biosafety of RAW Cells

Level 2. The cells contain the murine leukemia virus.

5 Conclusions

This chapter in general narrates the culture of the different continuous cell lines originating from various mammalian organs such as the breast, ovary, prostate, lung, liver, cervix, kidney, and blood. The cell lines discussed herewith include breast cancer cell lines (**MCF-7** and **MDA-MB-231** cells), ovarian cancer cell line (**CHO** cells), prostate cancer cell line (**LNcaP** and **PC3** cells), cancer cell lines originating from the female cervix (**HeLa** cells), lung cancer cell lines (**A549** and **BEAS-2B** cells), kidney cancer cell lines (**HEK-293** and **VERO** cells), liver cancer cell line (**HEPG2** cells), and blood cancer cell lines (**JURKAT** and **RAW** cells). All these

immortal cell lines grow well in laboratory conditions except JURKAT cells. All these cell lines are either adherent or loosely adherent in nature. However, for experimental purposes, one adherent cell line such as **CHO cells** can be converted into nonadherent cells that can be grown in large numbers in suspension culture. **Such cell lines are of significant industrial importance** for the generation of various recombinant proteins. In general, the culture of these cell lines not only helps to enhance the basic knowledge of growth/proliferation, survival, apoptosis, and other pathophysiological responses of these cells but ultimately leads to develop various anticancer drugs.

6 Cross-References

- ▶ Culture of Neuron and Glia Cells
- ▶ Isolation and Primary Culture of Various Mammalian Cells
- ▶ Mammalian Cell Culture Types and Guidelines of Their Maintenance
- ▶ Primary Culture of Immunological Cells
- ▶ Stem Cell Culture and Its Applications

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Stem Cell Culture and Its Applications

Radhashree Maitra

Contents

Abstract

Cells are the structural and functional unit of life. The dynamics of life encompass the continuous interaction of the cells with the environment and the energy balance that it maintains for sustenance. Eukaryotic organisms' cells have evolved with specific functional commitments and are at the terminal stage of differentiation. With a better understanding of cellular development and differentiation mechanism, it is now appreciated that cells in general have a certain degree of plasticity and can alter with changes in the internal and external

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_12-1

environment. Furthermore, a population of uncommitted cells is also found in eukaryotes. These cells can differentiate into various lineages as and when necessary and are denoted as **stem cells**. The stem cells are not only found in a rapidly dividing fertilized embryo (**embryonic stem cells**) but are also found in specific niches of adult organs (**adult stem cells**) and provide a repository of uncommitted cells with a high degree of plasticity. The primary function of these pluripotent stem cells is to maintain homeostasis with the ability to replenish lost or defunct cells. Stem cell research has made immense progress in the past decade and holds great promise in future regenerative medicine. In today's scenario, one of the most widespread uses of stem cells is bone marrow transplantation, and it has revolutionized the treatment of some cancers. Although stem cell therapy is yet to be introduced in many other clinical practices, it is progressively being evaluated as a treatment option for many present-day incurable diseases.

Keywords

Stem cells · Differentiation · Pluripotent · Reprogramming · Bone marrow

1 Introduction

Stem cells are an integral component of our system and continue to be so from the early stages of development to the end of life (Andrews et al. 2005). Essentially, stem cells are undifferentiated cells that have the unique property to develop into the specialized and committed cells of the organism's body (Kumar et al. 2010). Thus, stem cells play a vital role in the development, growth, maintenance, and repair of cells of various organs of the body. Stem cells provide foundational support to every organ and tissue in the human body. The highly specialized cells present in various tissue types originate from an initial pool of stem cells generated immediately after fertilization (Siminovitch et al. 1963). The living body continues to rely on stem cells to replace injured tissues and cells that are lost every day. Stem cells have two fundamental properties: 1) the ability to self-renew, dividing in a way that makes copies of themselves, and 2) the ability to differentiate, giving rise to the mature types of cells that make up our organ and tissues.

Stem cell research holds tremendous potential for the development of novel therapies for many serious diseases and injuries. Embryonic stem cells provide the platform for developing an in vitro model of mammalian development and represent a putative new source of differentiated cell types for cell replacement therapy (Keller 2005). Stem cell-based treatments have been established as a clinical standard of care for some conditions, such as hematopoietic stem cell transplants for leukemia and epithelial stem cell-based treatments for burns and corneal disorders, thus expanding the possibility of stem cell-based therapies (Daley 2012). In the recent past, scientists have gained sufficient experience with stem cell research to consider the possibilities of growing them outside the body for long periods of time. With such advances,

rigorous experiments can be conducted in such a way that specific tissues and organs can be grown with therapeutic competence.

Cell proliferation within a developing multicellular organism is an important characteristic that is followed by the process of differentiation and specialization. Differentiation takes place in steps. At each successive cell division and differentiation, the range of possible future identities for that cell lineage is progressively narrowed, until it culminates in a single cell type. Once a cell lineage has differentiated in the direction of a particular specialized cell type, all progeny cells are committed to becoming similar in all identities. Notably, a few groups of cells remain undifferentiated, and these are called stem cells. Embryonic stem cells have the potential to generate every cell type found in the body. Just as importantly, these cells can, under the right conditions, be grown and expanded indefinitely. Hence, these cells can serve as an important tool for researchers to learn about early developmental processes that are otherwise difficult to access. Such studies would help in establishing strategies that could lead to the development of therapies designed to replace or restore damaged tissues. Stem cells thus have the ability to recreate functional tissues.

Like all other types of stem cells, the adult stem cells (ASC) have in common two important characteristics. First, they are geared towards making identical copies of themselves for very long periods of time which usually spans throughout the life of the individual; this ability to proliferate is referred to as long-term self-renewal. Second, they give rise to mature cell types that have characteristic morphologies (shapes) from the organ they reside on and are capable of performing tissue-specific specialized functions. Commonly, an intermediate cell type or types are generated prior to fully differentiated stem cells and are termed as a precursor or progenitor cell. The partially differentiated progenitor cells found in adult tissues are often defined as pluripotent ASC that can divide and give rise to organ/tissue-specific differentiated cells. These pluripotent adult stem cells are termed “committed” cells that are already destined to differentiate along particular cellular pathways, although in rare cases some alterations in commitment can be observed and such changes need to be defined in a different cellular environment.

2 Types of Stem Cells

Stem cells are pluripotent cells found in all multicellular organisms. These cells retain the ability to renew themselves through mitotic cell division and can differentiate into a diverse range of specialized cell types (Becker et al. 1963). Two major categories of mammalian stem cells are (a) embryonic cells found in blastocysts of a developing embryo and (b) adult stem cells (ASC) found in adult tissues for constant replenishment of damaged tissues throughout the lifetime of a living being. In a developing embryo, stem cells can differentiate into specialized embryonic tissues, which differentiate into the specific lineage of cells with specialized cellular activities. Cellular repair and regeneration is an important process in adult organisms, which is accomplished by progenitor stem cells to maintain the turnover of damaged

and dying cells. This phenomenon is commonly observed in rapidly turned-over tissues like blood, skin, or intestine (Kumar et al. 2010). ASCs are in general undifferentiated cells found living within specific differentiated tissues in our bodies to generate new cells that can come to rescue to replenish any dead or damaged cells. The term "somatic stem cell" is sometimes used to refer to adult stem cells. ASCs are typically scarce in native tissues which has rendered them difficult to study and extract for research purposes. Resident in most tissues of the human body, discrete populations of ASCs generated cells to replace those that are lost through normal repair, disease, or injury. ASCs are found throughout the lifetime of an individual in tissues such as the umbilical cord, placenta, bone marrow, muscle, brain, fat tissue, skin, gut, etc. Adult stem cells were isolated and employed for blood products in the middle of the twentieth century, precisely in 1948. Twenty years later in 1968, this important technique was further expanded when purified ASC were prepared and used for clinical purposes, particularly for blood-related diseases.

Studies proving the specificity of developing ASCs are controversial; Some data specifically indicates that the adult stem cells can differentiate only to the cells of the tissue of origin while others have shown that the ASCs are more versatile and retain the power to differentiate into any tissue type. It is too early to accept either of the proposition with certainty and more experiments are needed for concrete confirmation. Stem cells are unique in their inherent characteristics to be transformed into specialized cells that are consistent with cells of various tissues such as muscles or nerves through cell culture, their use in medical therapies has been recognized and investigated. Interestingly when a stem cell divides, it can either remain a stem cell or turn into a differentiated cell, such as a muscle cell or a red blood cell, or other such types of cells. Thus, the populations of undifferentiated stem cells remain more or less constant during a major part of an individual's life.

Another subset of stem cells that has received considerable attention and exploration is cancer stem cells (CSC). Even when the cancer is diagnosed and treated at an earlier stage, some residual cells still remain and may cause tumor recurrence later with metastasis and aggressiveness. Growing evidence has implicated that these residual cells, which could be found during any stage of cancer progression that is responsible for causing the therapeutic resistance, possess stem-like properties/functions known as cancer stem cells. Hence, this population of cells represents the critical subset within the tumor mass in perpetuating the tumor, even after what seems to be effective therapy and leads to tumor aggression. In recent decades, the CSC theory generates much attention and excitement, whereby scientists believed this theory will revolutionize our understanding of the cellular and molecular events during cancer progression contributing to therapy resistance, recurrence, and metastasis. The CSC theory of cancer progression presents a tumor as a hierarchically organized tissue with a CSC population at the top rank in the hierarchy, which then generates the more differentiated bulk of the tumor cells with lower or limited proliferative potentials. Normal stem cells and CSCs have several commonalities that include self-renewing characteristics including heterogeneity and aberrant cell growth that may lead to benign and neoplastic tumors.

Cancer stem cells are generally characterized by the expression of stem cell-associated surface markers such as CD133, CD44, CD90, and side population cells (SP) by which they can be isolated and enriched in vitro and in vivo, although no single marker can be used to define the CSC populations. It is imperative that better understanding of CSCs characteristics, which alters in accordance with their micro-environment, often can be correlated to the different stages of cancer progression. It is thus very important to understand at the molecular level the strategies adopted by the CSC to remain re-renewable in an effort to better devise effective therapeutic strategies for harnessing the CSC population.

2.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) are derived from the undifferentiated inner mass cells of a human embryo. These cells are pluripotent, with the ability to divide and differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. It essentially means that the embryonic stem cells have the ability to mature into each of the more than 220 cell types found in the adult body as long as and when they are specified to do so. The time-bound activity of the specific signaling molecules is the major determinant in guiding the differentiation and growth of ESCs. Thus, embryonic stem cells are distinguished by two distinctive properties: their pluripotency, and their ability to replicate indefinitely. Pluripotency of the embryonic stem cells can be distinguished from adult stem cells that are primarily multipotent and can produce only a limited number of cell types. Embryonic stem cells can thus be utilized as useful tools for both research and regenerative medicine as these cells can divide limitlessly for a continuous supply of cells for research and clinical use.

2.1.1 Culture of Embryonic Stem Cells

Embryonic stem cells originate from human embryos that are 3–5 days old. In vitro fertilization is often employed to create and harvest these cells. Thus, the process involves fertilizing an embryo in the test tube within the laboratory settings instead of implantation inside the female uterus. Often these embryonic stem cells are also termed pluripotent stem cells. These cells are equipped with the ability to differentiate into virtually any other type of cell that is present within the body of the organism. One of the primary intentions of this investigation is to understand the exact mechanism of the process of differentiation from an embryonic stem cell to a fully differentiated cell of specific tissues and organs.

Scientists have been interested in determining the controlling factors of the process of differentiation. Many techniques have been developed by researchers to manipulate and direct the stem cells to a definitive lineage – a process termed directed differentiation. One of the pioneering efforts and accomplishments of isolating human embryonic stem cells (hESCs) from the inner cell mass (ICM) of human blastocysts was achieved by Ariff Bongso's group in 1994 (Bongso et al. 1994) (Fig. 1).

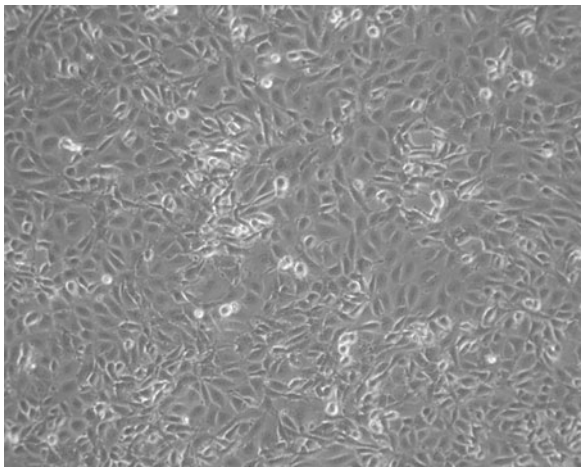

Fig. 1 Human embryonic stem cells in culture: Radhashree Maitra (Montefiore Medical Center, Bronx, NY)

2.1.2 Culturing Embryonic Stem Cells (Adopted from Philip H. Schwartz et al. (2011))

Reagents

- Dulbecco's modified Eagle medium: Ham's F12 (DMEM/ F12 + GlutaMAX. Invitrogen, #10565).
- Dulbecco's phosphate buffered saline (DPBS−−) without Mg^{2+} and Ca^{2+}.
- Dulbecco's phosphate buffered saline (DPBS++) with Mg^{2+} and Ca^{2+}.
- KnockOut™ Serum Replacement (KSR) (Invitrogen, #108280–028).
- 2-Mercaptoethanol, 55 mM (1000×) in PBS (such as Invitrogen, #21985–023).
- GlutaMax (100×) (Invitrogen, #35050).
- Human basic fibroblast growth factor (bFGF) (Stemgent, #03–0002).
- MEM-Nonessential amino acids (NEAA) 100× (10 mM) (Hyclone, #SH30238.01).
- Hybri-Max dimethyl sulfoxide (Sigma-Aldrich, #D2650).
- Water for embryo transfer (Sigma, #W1503, see Note 3).
- Fetal bovine serum (FBS) (Hyclone, #SH30070.03).
- Pen-strep 100× (optional) (Invitrogen, #15070–063).
- TrypeLE-Express (Invitrogen, #12604).
- Collagenase IV (20,000 U, Invitrogen, #17104–019).
- CF-1 mouse embryonic fibroblasts (ATCC, #SCRC-1040).
- Nikon Object Marker, catalog # MBW10020 (optional).
- 6-well vacuum gas plasma-treated tissue culture dishes (such as BD Falcon, #353046).
- Sterile nylon membrane syringe filter (Pall Life Sciences, #PN 4433).
- Nalgene freezing container (containing isopropanol).
- 20 μL pipette tips (Eppendorf and others).
- 150 mm Tissue culture dishes (TPP, #93150).

Media and Stock Solutions

- Human Basic FGF (bFGF) (10 μg/ml, 1 ml)
- Dissolve 10 μg human bFGF in 1 ml KSR.
- Aliquot in 50 μl samples.
- Store thawed aliquots at 4 °C for up to 2 weeks.
- Store frozen aliquots at −20 °C or − 80 °C for 6 months.
- Collagenase IV (200 U/ml, 100 ml).
- Dissolve 20,000 U of collagenase IV in 100 mL of DMEM/F12 + GlutaMax. This is usually ~1 mg/ml final concentration.
- Add to a 250 ml, 0.2 μm filter unit and filter sterilize.
- Aliquot in 5–10 ml in sterile tubes and store at −20 °C until use.
- KnockOut™ Serum Replacement.
- Aliquot as follows:
- Thaw 500 mL bottle at 4 °C and aliquot into sterile 50 mL tubes and store at −20 °C.
- Mix thoroughly when thawed, both for the initial aliquotting and when for use in media preparation.
- MEF Medium (500 ml):
  - Combine 440 mL DMEM/F12 + GultaMax, 50 mL FBS, 5 mL GlutaMax, 5 ml NEAA.
  - Sterile filter 2 μm. Store at 4 °C.
  - Warm to room temperature in the hood before use, discard unused medium after 2 weeks.
- Human Stem Cell (PSC) Medium (100 ml):
- Combine, in order, 78.8 mL DMEM/F12 + GlutaMax with 20 mL KSR, 1.0 ml, 100× NEAA, 100 μL bFGF stock solution, and 100 μL of 1000× 2-mercaptoethanol.
- Filter using 2 μm PES filter.
- Store at 4 °C when not in use and discard any unused medium after 2 weeks.
- Human PSC Cryopreservation Medium (10 ml, 2×).
- Combine 2 ml of human PSC medium, 6 mL of FBS, and 2 ml of DMSO.
- Mix thoroughly.
- Sterile filter using a syringe filter approved for use with DMSO (e.g., nylon membrane).
- Keep cold and use immediately. This is a 2× solution.

Procedure

These methods presume that all PSC culture is carried out in 6-well plates.

Preparation of Feeder Cell Stocks

- The traditional feeder cells are mitotically inactivated, low-passage mouse embryonic.
- fibroblasts, usually from CF-1 strain mice. These MEFs are seeded at a wide range of densities depending on the different PSC cell lines being grown. For

example, the original "H" series lines from WiCell were grown in the presence of MEFs seeded at 75,000 cells/cm^2. Many of the lines and others have been successfully grown on denser feeder layers. It will take some trial and error in your laboratory to determine the optimum density. Human-derived fibroblasts of various origins have also been successfully used as a feeder cell layer. Although not conclusively agreed upon, there is not much of an advantage of human versus mouse feeder cells. In general, some trial-and-error steps are necessary to determine the optimal feeder layer concentration.

A cryopreserved vial of mouse embryonic fibroblast has to be thawed quickly using a 37 °C water bath taking care that the cap is not submerged, followed by washing with 70% alcohol prior to moving it to the laminar flow hood. The contents are then carefully removed to a 15 ml centrifuge tube. 10 ml of warm MEF medium should then be added slowly and dropwise, while the tube is gently shaken.

- Aspirate the supernatant post-centrifugation for 5 mins at 200 × g followed by resuspension of the pellet in 5 ml of MEF medium.
- Add an additional 15 ml of MEF media after seeding onto a 150 mm tissue culture dish (TPP) with 0.1% gelatin coating. Rock the plate gently sidewise and back and forth in an incubator for even cell distribution.
- The cells have to be monitored daily. It usually divides extremely fast. Confluency can be reached in just 24 h.
- Once confluent the cells should be split at a 1:2 ratio using TrypLE Express. The media has to be aspirated out and the plate rinsed with 5 ml DPBS and add 10 ml of RT or 37 °C TrypLE Express.
- The cells start to lift off the plate within 24–48 h, the enzyme is next inactivated by adding 10 ml of MEF medium which has to be previously pre-warmed.
- Cells are next collected in a sterile conical tube and centrifuged for 5 min at 200 × g. Post-aspiration the cells are resuspended in 40 mL of medium and reseeded into 2–150 mm dishes. This step has termed passage I.
- The process is repeated with continuous monitoring and replating using MEFs until passage 5 is reached. In passage 5, the cells are lifted to irradiate them @ 3000 rads for the purpose of inactivation.
- At this stage, the cells can be frozen in DMEM supplemented with 30% FBS and 10% DMSO. Depending on the PSC line, the freezing density can be determined. Typically, feeder cells can be frozen in a single vial (~3.5 × 10^6/vial) to seed an entire 6-well plate.

Preparation of a Feeder Layer

- Coating the plate with 0.1%gelatin for 2–24 h before plating MEFs is necessary for the PSCs to attach. Plate the feeder cells 24 h before starting a PSC culture.
- A vial of irradiated MEFs cells is plated and seeded onto the plate in a MEF medium.
- MEFs are attached by overnight so the MEF medium can be aspirated out and rinsed with DPBS++. This should be followed by the addition of 1 ml/well PSC

medium. At least an hour is necessary for conditioning MEFs prior to seeding with the PSCs.

2.1.3 Thawing of Cryopreserved Pluripotent Stem Cells

- Often a growth lag is observed after thawing and plating PSCs, and may lead to a waiting period of several days before the colonies can be seen. Cultures during the lag period can be monitored microscopically under 4× magnification 24 h post-thawing and plating, but the medium should only be changed at least after 48 h. Usually floating debris and dead cells are observed upon thawing the cells, which is quite normal.
- The cells are thawed quickly, but gently shaking the vial and placing it in a 37 °C water bath and waiting until all ice has melted (about 60 s). Next, the vial is sprayed with 70% alcohol and dried with a Kimwipe.
- Remove the cells slowly and aseptically from the vial and place them in 15 ml conical tube. The entire process should be performed in a biosafety cabinet. Next, add 10 ml of room temperature PSC medium and tap gently.
- Centrifuge at 200 × g for 5 min.
- Aspirate out the supernatant.
- 3 ml of PSC medium is next added to the centrifuge tube, gently triturated, and transferred to one well of a 6-well dish that has been previously prepared with inactivated MEFs.
- The 6-well plate is then placed into the 37 °C incubator.
- 3–7 days have to be waited for the cells to attach. During this time, replace half of the medium every other day being careful not to aspirate out and lose the cells.
- The medium should be replaced every day starting from day 4 to day 7 since thawing the cells, most of the cells should appear to have been attached.

2.1.4 Passaging of Pluripotent Stem Cells

Dissociation with a single cell population is traditionally unhealthy for human PSCs. Thus, the most reliable method for passaging undifferentiated PSC cultures has been the manual breakup of the colonies. This method although exhausting is still recommended for beginners unless familiarity with the cell culture techniques is developed and can easily recognize the differentiation of cells in the cultures.

2.1.5 Mechanical Dissociation

There are various tools that can be used for mechanical colony disruption and are often an individual preference. It has been observed that needles and pipette tips are commonly used because of their low cost and easy availability.

- The culture should be evaluated daily under the phase-contrast microscope at 10X magnification.
- Split the cells onto 3–6 plates of the same size as the original culture, taking into account the density of the original culture. If desired, the cells can be plated onto different-sized plates or dishes, and in such case calculate the volume of media to be added based on the surface area of the plates.

- Remove the overtly differentiated colonies so as not to disturb these during the dissociation process.
- The medium from the dish is discarded and replaced with fresh PSC medium.
- The colonies are next dissected by hand, either under a low-power dissecting microscope or in a laminar flow hood.
- Manual passaging of human PSCs in culture can be achieved using a sterile needle or pipette or sterile scalpel for slicing the colonies into about 100 s pieces.
- Each colony should be broken and moved into suspension by moving the tip around and across each colony in a crosshatch or a spiral motion. Pipette tips are generally a better tool for this disruption than the needles due to their larger bore. As the colonies are relatively large at the time of passage, it is generally easy to observe individual colonies on the plate, and, with practice; the passaging of an entire dish can be achieved in less than 20 min.
- Post-colony disruption a 5 ml pipette can be used to transfer the culture medium containing the dissected colonies to a 15 ml or 50 ml conical tube. The plate is rinsed with 1 ml PSC media and then circulate the medium sequentially from well to well before adding it back to the same 15 ml tube.
- The volume of PSC media is brought up appropriately and cells in the tube are made ready for seeding new plates.
- The cell clumps are gently triturated with a sterile 10 ml pipette and divide the cell suspension into the prepared culture dishes on top of the feeder layer. Trituration should be performed gently. Avoid overtrituration as the goal is to achieve a relatively uniform distribution of the cell clumps without creating single cells.
- The newly seeded plates are placed in the incubator. The plates should be vigorously moved back and forth, side to side, and forward and backward to ensure even dispersion while being careful not to splash any medium onto the cover of the culture dish.

2.1.6 Collagenase Dissociation

Enzyme-mediated dissociation techniques vary between laboratories and generally follow various modifications of the original protocol. Exceptional care has to be exercised when using cultures that have been maintained by manual passaging. The cells cannot be passaged by enzymatic dissociation unless it is allowed to adapt to the new set of conditions. If proper care is taken, enzymatic passaging can provide a convenient and efficient way of maintaining PSC culture stocks.

- The culture medium is discarded.
- Followed by washing the culture with DPBS++.
- The cells are then treated with 2 ml per well 200 U per mL collagenase IV solution for 10 min at 37 °C. The culture must be checked under the microscope to observe the curling of the cells when the collagenase treatment can be terminated.
- The collagenase is next removed and replaced with 2 ml per well of PSC medium.
- A 5 ml pipette can be used next to gently dislodge the “good” colonies from the plate and transfer them to a 15 ml centrifuge tube. Alternatively, the differentiated

colonies can be carefully removed prior to treating the culture plate with collagenase.
- The cells are then gently triturated the clumps with a sterile 10 ml pipette and subsequently plated on a feeder layer of MEFs. Formation of single-cell suspension should be avoided and relatively uniform suspension of cell clumps should be achieved each containing several hundred cells.
- Finally, the cells can be divided into three to six dishes of the same size as the original culture, depending on the starting cell density. If different-sized dishes are used for plating, the appropriate cell dilution should be calculated based on the surface area.

2.1.7 Cryopreservation

To stabilize cultures with specific genetic characteristics at specific points in time, cryopreservation is essential. If cells are not cryopreserved, then it has to be continuously subcultured which will pose a serious threat. With each passaging and continuous subculturing, the cells may accumulate genetic changes and become heterogeneous. It is thus very important to use validated stock vials to initiate new experiments to maximize the long-term use of cell lines and minimize any experimental variation and ensure reproducibility.

The traditional method of cryopreservation of PSCs involves freezing the cells in large clusters with a medium containing FBS and DMSO. For PSCs, this technique has proven to be extremely almost inefficient with little or no cell recovery. If cryopreserved with media containing FBS and DMSO, the time from thawing the vial to having cultures ready for experimentation takes weeks to months. Scientists have now developed much more efficient techniques in conjunction with alternative culture systems.

- Cells are prepared for cryopreservation after it has reached the same stage at which it would be normally passaged.
- The culture media must be changed just before harvesting the cells.
- 1.8 ml cryogenic vials are first labeled with cell line name, date, and passage number.
- 2× stock cryopreservation medium is first prepared and stored on ice.
- The cells are next dislodged from the plate, mechanically, by using a sterile pipette tip. Some researchers may also prefer to treat the cell culture with 2 mL per well 200 U per ml collagenase IV in DMEM/F12 for 10 min at 37 °C.
- If the collagenase method is used, then the collagenase is removed and replaced with PSC medium (3 mL for each well of a 6-well dish).
- For each well of a 6-well dish, the cells are collected preferably in a 3 mL PSC medium and transferred to a 15 mL conical centrifuge tube.
- The collected cell suspension must then be centrifuged for 5 min at 200 × g. The supernatant is aspirated out, leaving a small volume of media just to cover the pellet.

- The cell pellet should then be gently resuspended in a conditioned PSC medium (usually 1.5 ml for each well of a 6-well dish or one-half of the final freezing volume). A 5 ml pipette may be used to gently triturate the clumps.
- An equivalent volume of ice-cold 2× cryopreservation medium is then added and mixed constantly by tapping the sides of the tube.
- 1.0 ml cell mixture is next added to each cryogenic vial (i.e., about three vials per well).
- Finally, the cell suspension is then rapidly transferred to the precooled (4 °C) Nalgene freezing container (containing isopropanol), and placed immediately in a freezer at −70 °C to −80 °C. The next day, the cryovials are transferred to liquid nitrogen for long-term storage.

2.1.8 Markers of Embryonic Stem Cells

Embryonic stem cells (ESCs) retain pluripotency and self-renewing ability due to both their inherent properties and the culture conditions in which they are propagated (Zhao et al. 2012). The expression of ESC markers is specific to their stages and differentiation process. In recent years, a wide range of cell surface markers has been identified as markers of undifferentiated ESCs, especially for the human species. Proteins involved in the cell signal pathways are also known to have important contributions to cell fate decisions. Unique gene expression patterns in ESCs can be used as the identification to distinguish a specific cell type from others. There are many molecules that can affect the pluripotency and self-renewal of ESCs. Identification, characterization, and categorization of these molecules will provide useful tools for the identification and isolation of the ESCs and subsequent ESC studies. A serious challenge is the overlap of ESC markers with those of tumor stem cells, making it difficult to use these markers for ESC identification and isolation. In addition, understanding the mechanisms that regulate the pluripotency of human ESCs (hESCs) remains a major challenge, as recent studies have shown that human and mouse ESCs differ in these mechanisms despite their similar embryonic origins (Prowse et al. 2007). Detailed knowledge of the mechanism of function of these markers is needed for the proper uses of ESCs and elucidation of the mechanisms governing the pluripotency and self-renewal of ESCs. The most common marker of ESCs that has been well studied by different laboratories includes **Oct 4, Nanog, SOX2, SSEA4,** and **Tra-1-60**. Antibodies to these proteins are commercially available.

- **Oct-4** (octamer-binding transcription factor 4) is involved in the self-renewal and replenishment of undifferentiated embryonic stem cells and is coded by the Pou5f1 gene. This gene is an important marker for undifferentiated cells. Oct-4 expression is required to be closely regulated; little or excess expression may cause unwanted differentiation of the cells (Niwa et al. 2000).
- **Nanog** is a homeobox gene coded by the NANOG1 gene. It is an important transcriptional factor that maintains the pluripotency of embryonic stem cells (ESCs) by actively suppressing the expression of the cell determination factors (Heurtier et al. 2019).

- **SOX2** (SRY (sex determining region Y)-box 2) belongs to the high mobility group box (HMG) gene and belongs to an important family of transcription factors that is critical in maintaining self-renewal, or pluripotency, of undifferentiated embryonic stem cells. (Rizzino 2009).
- **SSEA4** or stage-specific embryonic antigen-4 (SSEA-4) is encoded by the FUT4 (Fructosyltransferase 4) gene and is essentially a glycosphingolipid. SSEA4 is often expressed by ESCs. It is also expressed in certain cancers and used as a prognostic marker (Truong et al. 2011).
- **TRA-1-60** or T cell receptor alpha locus is expressed by ESC. This protein is expressed on the cell surface and plays an important role in cell differentiation. It is alternatively known as podocalyxin and is encoded by PODXL Gene (Schopperle and DeWolf 2007). It has been found to be expressed in embryonic cancers.

2.1.9 Usage of Embryonic Stem Cell

Research with embryonic stem cells reveals the possibility of implementing treatments of life-threatening human ailments and alleviating the cost and disease burden. Diseases like as type I diabetes, Parkinson's disease, heart disorders, and spinal cord damages are worth mentioning. The dual characteristics of the embryonic stem cells to proliferate indefinitely and to differentiate to mature tissue types can serve as a source of limitless supply of tissue-specific cells that can be therapeutically employed for cell therapy procedures with the goal to regenerate functional organs. However, there are several hurdles to be overcome for the successful clinical utilization of these cells. Furthermore, they can be employed for synthesis of organoids or development of organs like liver which can be used for transplantation. The ESC holds great promise with immense clinical application in the future. There are ethical issues and skepticism which has been the main hindrance in the stem cell-related research (Leventhal et al. 2012).

2.2 Neural Stem Cells

Neural stem cells (**NSCs**) are self-renewing tissue-specific stem cells found in both the fetal and adult nervous systems. NSCs were first generated from radial glial cells. Neural stem cells exist in the adult nervous system of all mammals as well as in the developing mammalian nervous system. Primitive embryonic stem cells can serve as a good source to generate neural stem cells. NSCs primarily differentiate into neurons, astrocytes, and oligodendrocytes and can be used to study the nervous system. Some neural progenitor stem cells persist within and are confined to specific regions in the brain. Neural stem cells are found to be present in the brain for the entire life. Their proliferation and differentiation are strictly balanced by intrinsic and extrinsic regulators (Jobe et al. 2012). The capability of culturing neural stem cells that are derived from embryonic stem cells, especially from induced pluripotent stem cells in vitro, makes it possible to provide an unlimited source for generating neurons, astrocytes, and oligodendrocytes, which can be used in neurological

disease modeling, neurotoxicological studies, and in cell replacement therapies for neurological diseases *(Yang et al. in Principles of Regenerative Medicine, 2008)*. Experimental evidence indicates that the mammalian central nervous system development undergoes both asymmetric divisions to give rise to progenitors (IPCs) as well as symmetric divisions to expand the NSC pool. The neurons, astrocytes, and oligodendrocytes differentiate from the neuronal stem cell in a temporal sequence.

Utilizing neural stem cells as a model for developmental neurotoxicity assessment has recently received broad attention, and the applications have been growing. Extensive knowledge regarding the characteristics of neural stem cells, specifically the controls of their proliferation and differentiation is necessary before the full potential of neural stem cells can be utilized. Importantly, the differentiation pattern that is available to the daughter cells has to be delineated. The development of a profiling pattern of the neural stem is urgently needed to establish a standardized protocol of testing for the assessment of possible neurotoxicity. The regulatory mechanisms determining the process of self-renewal and fate specification developmental features of NSCs have contributed tremendously to our understanding of neuron-related developmental diseases as well as human brain development in general. Additionally, this information has guided scientists to refine protocols for pluripotent stem cell differentiation into specific nervous system-related cell types for disease modeling as well as to modulate clinical approaches.

2.2.1 Preparation of Neural Stem Cells

Starting from Human Pluripotent Stem Cells (Adopted: Pistollato et al. 2017)
Generation of embryoid bodies (EBs) (Days 0 → 1) The procedure requires significant manual command and precision. Equal-sized fragments of the HiPSC colony are essential to obtain homogenous embryoid bodies (EBs) in the next steps. Colonies with large cytoplasmic fractions and small nucleoli that are not morphologically identical should be discarded.

- The hiPSC medium (3 ml/60-mm Petri dish) should be replenished before cutting the undifferentiated hiPSC colonies to about 1 mm in diameter under sterile conditions.
- 1 ml syringe with a 30G needle can be used to cut the undifferentiated colonies into fragments of approximately 200 μm x 200 μm. A dissecting microscope at 4X magnification is used within a laminar flow cabinet at room temperature during the operation.
- A 200 μl pipette is employed to detach the colony fragments from the dish surface by gently pipetting the medium underneath to lift the pieces of the cell colony.
- All of the detached fragments along with the media are transferred into a 15-ml tube using an appropriately sized pipette.
- The culture plate is then rinsed with 2 ml complete hiPSC medium to collect all fragments.
- The collection is followed by centrifugation at 112 x g for 1 min.

- Complete iPSC EB media is aspirated onto the supernatant to gently resuspend the fragments.
- The colony fragments are next plated in a 60 mm ultralow attachment Petri dish and incubated overnight at 37 °C and 5% CO_2.
- Next day (Day 1), the EBs and the culture media must be transferred into a 15 ml tube.
- The EBs collected in the 15 ml tube should be centrifuged at 112 x g for 1 min.
- The supernatant is aspirated out with care not to lose any EBs which should then be gently resuspended in a complete hiPSC EB medium.
- The EBs are then replated onto a new 60-mm ultralow attachment Petri dish (5 ml/60 mm Petri dish).
- The Petri dish should be next incubated overnight at 37 °C and 5% CO_2.

On Day 1, the dishes are coated with basement membrane matrix (e.g., matrigel, referred to hereafter as "standard matrix") or any other suitable protein substrate (e.g., laminin).

- The standard matrix is stored at −80 °C in 200 µl aliquots using cold 1.5 ml tubes and cold 10 ml pipettes.
- 200 µl standard matrix is thawed on ice.
- 200 µl standard matrix is diluted in 20 ml DMEM/F12 medium (1:100 dilution).
- 60 mm Petri dishes are coated with the standard matrix solution typically 5 ml per dish.
- The coated dishes are then incubated at 37 °C overnight. Note: These dishes will be used to plate the EBs (about 50 EBs/dish) and to generate the neuroepithelial aggregates (rosettes).

Generation of neuroepithelial aggregates (rosettes) (Days 2 → 7)

- **On Day 2,** the standard matrix coating solution should be removed from the 60-mm Petri dishes and refilled with 5 ml/dish of complete neuroepithelial induction medium (NRI) without any prior rinsing off the plate.
- The floating EBs are next transferred to the coated dishes (~50 EBs/dish) using a 200 µl pipette under 4x magnification (microscope) in a laminar flow hood.
- It is extremely important to select homogenous, medium-sized EBs (~200–300 µm in diameter). Very small EBs will not survive during neuroectodermal differentiation, while very large ones tend to undergo core necrosis.
- The dishes are then incubated at 37 °C and 5% CO_2.
- **On Day 3**, the dishes should be observed under the microscope at 10x magnification to ensure that the EBs are attached properly.
- Complete NRI medium is used to perform a total medium change. The process should be done carefully and gently.
- Every alternate day, the NRI culture media is changed for up to Day 7. At this point, the neuroepithelial aggregates (rosettes) should become visible.

- **On Day 7**, the standard matrix or laminin should be coated onto the experimental plate or 96-well plates (100 μl/well), 24-well plates (250 μl/well), 12-well plates (500 μl/well), 60 mm Petri dishes (4 mL/dish).
- The matrix-coated plates/dishes are incubated for at least 2 h at 37 °C and 5% CO_2.

Rosette dissociation and neuronal differentiation (Days 8 → 28) Caution: This procedure will require good manual skills and precision. To avoid collecting mesodermal and endodermal cells, only ectoderm rosette-like structures should be dissociated and collected.

- **On Day 8, t**he rosette-like structures are cut into fragments using a stereoscopic microscope at 10X magnification in sterile conditions. 1 ml syringe with a 30G needle should be used. The rosettes tend to loosen from the culture dish when touched with the needle; hence, caution should be exercised.
- The detachment of the rosette fragments is completed using a 200-μL pipette.
- The dish is transferred under the laminar flow hood and the rosette fragments are collected employing a 5 ml pipette along with the culture NRI medium into a 15 ml conical centrifuge tube. The dish should be rinsed with 2 ml NRI medium for complete recovery of all fragments.
- The rosette fragments are next centrifuged at 112 x g for 2 min and the supernatant is aspirated out.
- Next the pellets are gently resuspended in 1 ml, 1x DPBS (without calcium and magnesium), the rosette fragments are aspirated up and down using a 1000-μl pipette to ensure partial dissociation.
- About 4 ml of complete NRI media should be added followed by cell counting using trypan blue on a hemocytometer or a digital cell counter slide. 20 μl of cell suspension is added to 20 μl Trypan blue to prepare a 1:1 dilution before counting. This step will not be of any relevance if the cells cannot be brought to a single-cell suspension. If the rosette fragments are not observed to be completely dissociated, the rosette fragments derived from about 50 EBs/60-mm dish should be resuspended in 50 ml complete NRI media.
- The standard matrix (or laminin) coating solution is aspirated out of the Petri dishes, plates, and/or MEA chips. Caution should be taken to not let them dry.
- Next the cells are plated in a complete NRI medium at a concentration of about 15,000 cells/cm^2.
- The plates are incubated overnight at 37 °C and 5% CO_2.
- **On Day 10**, a total media change is performed and replenished with a complete neuronal differentiation medium (ND).
- The complete ND medium is changed and replenished twice a week until Day 28.

2.2.2 Expansion and Differentiation into Mixed Neurons and Glia

NOTE: The procedure described below is employed for expanding and maintaining NSCs derived from rosette fragments. This technique allows for incrementing the number of cells for differentiation and chemical testing.

- 60 mm Petri dish (or a T-25 flask) is coated with 5 mL of standard matrix DMEM/F12 coating solution and incubated for at least 2 h at 37 °C and 5% CO_2
- The rosette fragments that were previously derived are placed in a conical 15 ml tube and centrifuged at 112 x g for 2 min.
- Next the pellet is gently resuspended in a 5 ml neural induction medium (NIM).
- The cells are next transferred onto a standard matrix-coated 60 mm Petri dish/flask culture rosette-derived NSCs in the presence of NIM, refreshing the medium every other day until the cells reach confluence.
- After confluency is achieved, the NSCs are passaged as detailed in the following steps.
- The NSCs should be passaged at least once a week using freshly coated dishes, flasks, or plates.
- The complete NIM media is aspirated out, and the NSCs are gently rinsed with DPBS (without calcium and magnesium).
- 1.5 ml of 0.05% trypsin-EDTA pre-warmed to 37 °C should be added to the 60 mm Petri dish (or T- 25 flask) containing the cells and placed in the incubator for 1 min only.
- The flask is tapped on the sides gently to detach the cells.
- 1.5 ml trypsin inhibitor pre-warmed to 37 °C is added and the cells are transferred to a 15-ml centrifuge tube.
- An equal volume of NIM is added to the Petri dish or the flask to rinse the cells.
- The cells are next collected in a 15-mL tube and centrifuged at 130 x g for 3 min.
- The supernatant is removed and the cells are gently resuspended in 1 ml complete NIM using a 1 ml pipette.
- The cell suspension can be further diluted (10 to 100 times as necessary) in 3 or 4 ml complete NIM and the cells are counted after trypan blue staining and counted on an automated cell counter.
- The NSCs are next plated onto the 60-mm Petri dish or flask usually at a density of about 50,000 cells/cm^2.
- A total media change must be given every 48 h with complete NIM media.

2.2.3 Cryopreservation and Thawing NSCs

This is an important step to be followed carefully if frozen cells are re-thawed.

- The NSCs cells at the time of passaging are collected and centrifuged at 130 x g for 3 min.
- The NSCs are very gently resuspended in 3 x 10^6/ml of freezing medium.
- The cells are then aliquoted in suitable vials for cryopreservation (about 0.5 mL = 1.5 x 10^6/vial).
- The vials are placed in a container filled with isopropanol and stored at −80 °C for a minimum of 2 h and a maximum of up to 2 weeks.
- The vials should then be transferred to the vapor phase of a liquid nitrogen tank.
- When cell culture revival is necessary, the frozen vial is quickly thawed in a water bath at 37 °C.

- Using a 1000-μL pipette the cells are gently collected in a 7 ml pre-warmed complete NIM in a 15-ml tube.
- The 15 ml tube is next centrifuged at 130 x g for 3 min to collect the cell pellet.
- The supernatant must be removed and the cell pellet is gently resuspended in 1 ml complete NIM with the help of a 1000 μl pipette.
- The cells are resuspended in 3 or 4 ml complete NIM and quantified by counting the cells using trypan blue and an automated cell counter.
- To initiate a fresh culture. The NSCs are plated in a coated 60 mm culture dish or flask at a density of about 50,000 cells/cm^2.

2.2.4 Characterization of Neuronal and Glial Cells

Differentiated neuronal and glial derivatives can be characterized using various techniques, such as quantitative real-time PCR and/or immunohistochemistry and high content imaging.

2.2.5 Quantitative Real-Time PCR Analyses (Schmittgen and Livak 2008)

- The hiPSC colony fragments, EBs, and/or NSCs are centrifuged at 130 × g for 3 min.
- The cell pellet is then resuspended in 100 μl, cold RNA lysis buffer usually provided in the kit for RNA extraction.
- The process can be alternatively achieved by collecting the neuronal/glial derivatives directly from the plates by aspiration of the medium and adding the cold RNA lysis buffer directly to the wells to collect the lysed cells.
- The RNA isolation is then performed following the kit manufacturer's instructions.
- The RNA is quantified by nanodrop measurements or other measurement techniques.
- 500 ng of total RNA is used to prepare cDNA by reverse transcription.
- A qPCR reaction mix is prepared in duplicate using the appropriate master mix and primer sets.
- The mean fluorescence is recorded in real time: Typically, 40–45 cycles are run with primers annealing temperature set at 60 °C.
- The relative expression of RNA transcripts is determined by normalization to the expression of the housekeeping gene which might be GAPDH or β-actin as a reference gene. Finally, the expression of the desired gene is determined by ΔΔCt method. Alternatively, another suitable method for quantifying gene expression can be used.

2.2.6 Immunocytochemistry and High-Content Imaging (Obernier and Alvarez-Buylla 2019)

- All the colonies including hiPSC, NSCs, and/or neuronal/glial derivatives should be first fixed for 15 min at room temperature with cold 4% paraformaldehyde.
- The cells are then carefully washed with 1X PBS and the plates can be stored at 4 °C for up to 1 month.

- Just prior to staining, the fixed cells have to be permeabilized in permeabilization buffer (1x DPBS containing 0.1% triton-X-100 and 3% BSA) for 15 min at room temperature.
- The permeabilization buffer is aspirated out and the cells are incubated in blocking buffer (3% BSA/1X DPBS) for 15 min at room temperature, a step important to prevent the nonspecific binding of antibodies.
- After 15 min, the blocking buffer is removed by quick centrifugation, and the cells are incubated with primary antibody diluted in blocking buffer overnight at 4 °C.
- The next day, the antibody solution is removed and the stained cells are washed 3 times with cold 1x PBS at 4 °C by centrifugation.
- The cells are incubated at room temperature for 45 min in a blocking buffer containing fluorochrome-conjugated secondary antibodies, followed by counterstaining the nuclei with DAPI dye.

The mean fluorescence intensity should next be quantified, and the relative percentages of cell types should be determined utilizing a suitable high-content imaging platform: There is a necessity to incubate some cells with secondary antibodies alone to estimate the background level of the fluorescent intensity. The expression of PSC-specific markers, such as SSEA4, should be assessed by flow cytometric analysis of live undifferentiated hiPSCs. Further, some of the Undifferentiated hiPSC colonies can be analyzed for alkaline phosphatase activity with BCIP/NBT kits, following the manufacturers' instructions. Additionally, reverse phase protein array (RPPA) assays and analyses should be performed.

2.2.7 Electrophysiological Measurements (Vassallo et al. 2017)

- The dissociated rosette fragments are plated after seven divisions or NSCs derived from rosettes on coated multielectrode arrays are plated in a complete ND medium at a cell concentration of approximately ~1×10^5 cell.
- The cells are allowed to differentiate for three continuous weeks in a complete ND medium, with a routine media change twice a week.
- Once the differentiation is completed, the MEA chips are sealed with a semipermeable membrane under a laminar flow hood to keep the cultures sterile for repeated measurements.
- One of the electrodes is next replaced with one ground reference so that recordings from the remaining electrodes can be collected properly.
- The mean firing rate (MFR; the number of spikes/min) is recorded using an MEA amplifier with the integrated temperature process control that is adjusted to 37 °C and 5% CO_2.
- The relevant peaks are detected from the MEA raw data using a threshold limit of -4.7σ (σ represents the standard deviation of the basal noise).
- The recorded data can then be appropriately processed using suitable software.

2.2.8 Neural Stem Cell Marker

Marker-specific identification of neural stem cells is essential for studying the basic mechanisms of the generation of cellular diversity in the CNS, upon which therapeutic treatments for CNS injuries, degenerative diseases, and brain tumors may be based (Kaneko et al. 2000). Progress has been made in delineating the properties of adult NSCs, specifically the lineages they develop into as well as the signaling pathways that determines their behavior, but as of the date the extrinsic and/or intrinsic factors that promote quiescence/dormancy and activation of NSCs remain inconclusive and the detailed understanding of the mechanisms that sustain the NSC pool while ensuring life-long neurogenesis is still lacking. Furthermore, a significant hurdle is the heterogeneity of the population of primary progenitors/NSCs in the mammalian brain is yet to be overcome. The consequences of the heterogeneity in terms of affecting the NSC function are still obscure (Obernier and Alvarez-Buylla 2019). Most utilized neural stem cell markers include **Nestin, SOX2, HES 1, and Notch1** while many more are being investigated.

- **Nestin** (an acronym for neuroectodermal stem cell marker) is a type VI intermediate filament protein that in humans is encoded by the NES gene. Nestin functions in the development of neurons.
- **SOX2** (SRY (sex determining region Y)-box 2) belongs to the high-mobility group box (HMG) gene functions as a transcription factor that is essential for maintaining self-renewal or pluripotency and is expressed in multipotent neural stem cells at all stages of human life (Ellis et al. 2004).
- **HES1** (hairy and enhancer of split-1) is a transcription factor that is encoded by the Hes1 gene and is one of the seven members of the Hes gene family. Hes genes code nuclear proteins that suppress transcription (Andrews et al. 2005) It is expressed in both neuroepithelial cells and radial glial cells and is involved in maintaining but not in creating neural stem cells.
- **Notch 1** is a single-pass transmembrane receptor encoded in humans by the NOTCH1 gene. Several developmental processes controlling cell fate decisions are controlled by proteins that can be grouped as Notch family of proteins. Notch 1 promotes the differentiation of progenitor cells into astroglia (Tanigaki et al. 2001). Notch 1, when activated before birth, induces radial glia differentiation, but postnatally induces the differentiation into astrocytes (Chambers et al. 2001).

2.2.9 Usage of Neural Stem Cells

Neural stem cells have been shown to engage in the migration and replacement of dying neurons. Cell death is a characteristic of acute CNS disorders as well as neurodegenerative disease. The cell loss is further amplified by the lack of regenerative abilities for cell replacement and repair in the CNS. One way to circumvent this is to use cell replacement therapy via regenerative NSCs (Xu et al. 2011). Neuronal precursors (also called neuroblasts) divide and give rise to nerve cells (neurons) of various types. Glial precursors give rise to astrocytes or oligodendrocytes. Astrocytes are a kind of glial cells, which lend both mechanical and metabolic support to neurons; they make up 70–80% of the cells of the adult brain. Oligodendrocytes, on

the other hand, make myelin, the fatty material that forms the sheath of the neuronal axons and speeds nerve transmission. Under in vivo conditions, neuronal precursors do not give rise to glial cells and glial precursors do not give rise to neurons. In contrast, a fetal or adult CNS (central nervous system – the brain and spinal cord) stem cell may give rise to neurons, astrocytes, or oligodendrocytes, depending on the signals it receives and its three-dimensional environment within the brain tissue (Ellis et al. 2004). It is now widely accepted in the scientific world that the adult mammalian brain has a niche of neuronal stem cells. However, there is no consensus about how many populations of CNS stem cells exist, how they may be related, and how they function in vivo. Notably, the role of NSCs during diseases is now being elucidated by several research groups around the world. The responses during stroke, multiple sclerosis, and Parkinson's disease in animal models and humans are part of the current investigation. The results of this ongoing investigation may have future applications to treat human neurological diseases (Fig. 2).

The flow chart (above) details the process of differentiation of human pluripotent stem cells (hPSCs) to neuronal cells. The cell size at the various stages of development along with the type of growth media that facilitates the differentiation is depicted.

2.3 Mesenchymal Stem Cells

Human MSCs (hMSCs) are non-hematopoietic, multipotent stem cells with the capacity to differentiate into mesodermal lineages such as osteocytes, adipocytes, and chondrocytes as well ectodermal (neurocytes) and endodermal lineages (hepatocytes) (Ullah et al. 2015). Mesenchymal stem cells or MSC are often referred to as cells isolated from stroma, the connective tissue that surrounds other tissues and organs. These cells are often referred to as "stromal cells" which is a more accurate annotation. MSCs were first isolated from the bone marrow and were shown to be capable of making bone, cartilage, and fat cells. It is generally agreed that in an embryo a mesenchymal stem cell is a pluripotent progenitor cell that divides many times and whose progeny eventually gives rise to skeletal tissues: cartilage, bone, tendon, ligament, marrow stroma, and connective tissue (Caplan 1991). In 1959, a bone marrow (BM) transplant was applied for the first time in patients after confirming its therapeutic effects in dogs, as a hematopoietic stem cell-based therapy (Abdal Dayem et al. 2019). Alexander Friedenstein first identified MSCs as colony-forming unit fibroblast and osteoprogenitors with a fibroblast-like shape, that can grow in adherent cell colonies, and were distinctly different from the hematopoietic stem cell (HSC). In 2007, Sacchetti et al. documented the self-renewal potential of osteoprogenitors in BM sinusoids by showing their ability to organize specifically in the hematopoietic microenvironment (Sacchetti et al. 2007). In subsequent years, the MSCs were successfully grown from other tissue types, including adipocytes and cord blood cells. MSCs are proposed to have stem cells, with immunomodulatory properties, and are currently being tested as treatments for several types of disorders, although the benefits are yet to be established. Scientists do not fully understand

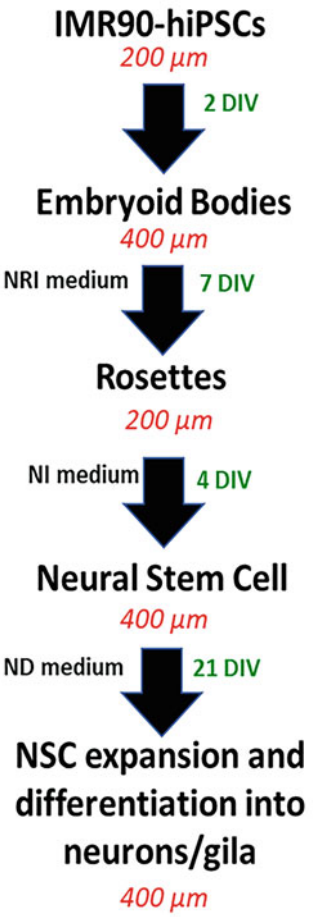

Fig. 2 Expansion and differentiation of hiPSC cells neurons/glia

whether these cells are stem cells or what types of cells they can generate. Scientists do agree that not all MSCs are identical and that their characteristics depend on where in the body they come from and how they are isolated and grown. MSCs do indeed possess a diverse range of therapeutic applications.

2.3.1 Preparation of Mesenchymal Stem Cells

MSCs are multipotent stem cells that can be easily isolated from various tissues and organs of the human body, such as bone, fat tissue, cartilage, hepatic tissue, blood, and muscle. Crisan et al. 2008 have reported that irrespective of the tissue origin, long-term cultured perivascular cells retained myogenic properties; exhibited at the clonal level osteogenic, chondrogenic, and adipogenic potentials; expressed MSC markers; and migrated in a culture model of chemotaxis. MSC markers were found to be expressed at the surface of native, perivascular cells. Thus, blood vessel walls

may harbor a reserve of progenitor cells that may be integral to the origin of the elusive MSCs and other related adult stem cells. MSCs can be autologous (same patient-derived) or allogeneic (derived from another patient), which differentially influences the clinical application of MSCs in the various appropriate clinical settings (Trounson & McDonald 2015).

2.3.2 Mesenchymal Stem Cell Isolation from Mouse Bone Marrow (Adopted: Soleimani and Nadri 2009)

Materials

Reagents

- DMEM with 2 mM L-glutamine and without ribonucleosides and ribonucleotides
- FBS
- 0.25% trypsin/1 mM ethylenediaminetetraacetic acid
- Hanks' balanced salt solution
- Streptomycin
- L-glutamine
- Penicillin
- $NaHCO_3$
- FBS

Procedure

- Mice (Balb/c, 6–8 weeks old) are euthanized by cervical dislocation to harvest the bone marrow. The animal skeleton is washed with 70% ethanol, an incision is made around the perimeter of the hind limbs at the point where it is attached to the trunk. Initially, the skin is removed by pulling toward the skin by foot, which is cut at the anklebone. Further contact of the hind limb with the animal's fur is thus eliminated, which can be a potential source of contaminating bacteria. The hind limbs are dissected from the trunk of the body by cutting along the spinal cord with care not to damage the femur under the laminar flow hood. The limbs are preserved on ice in DMEM supplemented with 1Xpenicillin/streptomycin while awaiting further dissection.
- The bone marrow (BM) is harvested in a hood using a proper sterile technique. The ends of the tibia and femur are incised just below the end of the marrow cavity using a pair of sharp blades. A 27-gauge needle attached to a 10 ml syringe containing complete media has to be inserted into the spongy bone by the removal of the growth plate. The marrow plug is flushed out through the cut end of the bone with 1 ml of complete media and collected in a 10 ml tube on ice.
- Strong flushing is necessary during marrow cell preparation.
- The cell suspension should be filtered through a 70-mm filter mesh to remove any bone pieces/spicules or muscle and cell clumps. The yield and viability of cells is determined by Trypan blue exclusion and counting on a hemocytometer. Typically, 70 x 10^6 BM cells are obtained from one donor.

- The freshly isolated BM cells are next cultured in 95-mm culture dishes using 1 ml complete medium and plated at a density of 25 x 10^6 cells per ml.
- The plates are incubated at 37 °C with 5% CO_2 in a humidified chamber avoiding any disturbances. The nonadherent cells that accumulate on the surface of the dish are removed after 3 h; the plate is replenished with a fresh aliquot complete medium.
- Second round of media change is performed after an additional 8 h of culture, and another aliquot of 1.5 ml fresh complete medium is added. This step is repeated every 8 h for up to 72 h of initial culture.
- It is important to exercise caution when changing the media which should be done very slowly and gently to avoid unwanted lifting of the MSCs growing in the culture media.
- The adherent cells (passage 0) should be washed with phosphate-buffered saline, and fresh media is replaced every 3 to 4 days for healthy growth. It is normal to observe individual cells as adherent spindle-shaped cells under phase-contrast microscopy which may appear around the third day of culture.
- The culture generally becomes (65–70)% confluent within 4 to 8 days, the culture achieves full confluence within 2 weeks. At this stage, the cultures typically exhibit two characteristics: first, plates may contain distinct colonies of fibroblastic cells that vary in size; and second may contain very small numbers of hematopoietic cells interspersed between or on the colonies.
- After 2 weeks of initiating culture, the cells should be washed with phosphate-buffered saline, and cells are lifted by incubation in 0.5 ml of 0.25% trypsin/1 mM ethylenediaminetetraacetic acid for 2 min at room temperature. The trypsin is neutralized after 2 mins by adding 1.5 ml complete medium, and all lifted cells are transferred to a 25-cm^2 flask. The non-lifted cells should be discarded.
- The time and temperature of passaging are very critical and should be very carefully monitored (if the time and temperature were higher than 2 min and 25 °C, respectively, non-MSCs together with mMSCs would be lifted from plastic culture dishes).
- The culture media should be replaced every 3 days (replacing with 6 ml medium each time). Typically, cell confluence should be achieved within 7 days.

Timing

- Day 1, Steps 1–5: harvesting, seeding, and change of media of bone marrow cells
- Day 2–3, Step 5: slowly change the media of culture.
- Day 4–14, Steps 6 and 7: change the medium every 3 to 4 days.
- Day 14, Step 8: subculturing adherent bone marrow cells.
- Day 15–21, Step 9: obtain a purified population of mesenchymal stem cells with spindle-shaped morphology that appears to gradually increase on days 4 to 10.
- 21 days of culturing marrow cells.

2.3.3 Markers of Mesenchymal Stem Cells

Flow cytometry analysis demonstrated that the cluster of differentiation markers currently used to define MSCs, such as CD105, CD166, CD90, CD44, CD29, CD73,

and CD9. Many of these markers are also expressed by fibroblasts. Recently, real-time polymerase chain reaction confirmed that expression of CD106, integrin α11, and insulin-like growth factor-2 in MSCs was at least tenfold higher than in fibroblasts, whereas expression of matrix metalloproteinase 1 and matrix metalloproteinase 3 was almost 100-fold lower. It is relatively easy to recognize MCSs from hematopoietic stem cells as the former does not express the CD45 cell surface protein marker. The MSCs also have an upregulation of CD106 as confirmed by immunostaining and live cell flow cytometry analysis and this can further help to distinguish itself from fibroblasts.

2.3.4 Use of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a subset of adult stem cells that may be particularly useful for stem cell-based therapies for three reasons. **First**, MSCs have been isolated from a variety of mesenchymal tissues, including bone marrow, muscle, circulating blood, blood vessels, and fat, thus making them abundant and readily available (Deans and Moseley 2000; Zhang et al. 2009). **Second,** MSCs can differentiate into a wide array of cell types; including osteoblasts, chondrocytes, and adipocytes. This suggests that MSCs may have broader therapeutic applications compared to other adult stem cells. **Third,** MSCs exert potent paracrine effects enhancing the ability of injured tissue to repair itself. In fact, animal studies suggest that this may be the predominant mechanism by which MSCs promote tissue repair.

The mesenchymal stem cells are universal in their presence and are found throughout the body's organs. Those harboring the bone marrow can be differentiated to become bone, cartilage, fat, and muscles. Notably, the MSCs are found to be able to alter immune functions when studied in several experimental models. These abilities have created considerable interest in developing ways of using mesenchymal stem cells to treat a range of musculoskeletal abnormalities, cardiac diseases, and some immune abnormalities such as graft-versus-host disease following bone marrow transplant. Other stem cell treatment modalities are in the early stages of development and hold great promise for the future.

MSC-based therapy has been found to cause certain paracrine effects in angiogenesis, antiapoptotic, and immunomodulatory processes. MSCs are known to influence innate and specific immune cells. MSCs produce many molecules having immunomodulatory parameters. These include prostaglandin E2 (PGE2) (Spaggiari et al. 2009), nitric oxide (Ren et al. 2010), indoleamine 2,3-dioxygenase (IDO), IL-6, and other surface markers – FasL (Akiyama et al. 2012) PD-L1/2. MSCs insert an effect on macrophages, neutrophils, NK cells, mast cells, and dendritic cells in innate immunity. MSCs are also capable to migrate to the site of injury, where they polarize through PGE2 macrophages in the M2 phenotype which is characterized by an anti-inflammatory effect.

MSCs when cultured can secrete hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF) (Nagaya et al. 2005). In a rat model of myocardial ischemia, injection of human bone marrow-derived stem cells upregulated cardiac expression of VEGF, HGF, bFGF, angiopoietin-1 and angiopoietin-2, and PDGF (Yoon et al. 2005). In swine, injection

of bone marrow-derived mononuclear cells into the ischemic myocardium was shown to increase the expression of VEGF, enhance angiogenesis, and improve cardiac performance (Tse et al. 2007). Bone marrow-derived stem cells have also been used in several small clinical trials with conflicting results. In the largest of these trials (REPAIR-AMI), 204 patients with acute myocardial infarction were randomized to receive bone marrow-derived progenitor cells versus placebo 3–7 days after reperfusion. After 4 months, the patients that were infused with stem cells showed improvement in left ventricular function compared to control patients. At 1 year, the combined endpoint of recurrent ischemia, revascularization, or death was decreased in the group treated with stem cells (Leventhal et al. 2012).

2.4 Hematopoietic Stem Cells

The first characterization of hematopoietic stem cells started when animals given lethal doses of irradiation suffered bone marrow failure, and this failure could be reversed by injection of nonirradiated bone marrow cells (Ford et al. 1956). The concept of multipotential hematopoietic progenitors derived from the first quantitative experiments on bone marrow restoration indicated that limiting numbers of bone marrow cells can give rise to clonal colonies of myeloid-erythroid cells. Essentially, these hematopoietic stem cells give rise to other blood cells through a process termed hematopoiesis. In embryonic development, this is derived from the mesoderm layer of the embryo. In adults, this process occurs in the red bone marrow. It is interesting that the generation of hematopoietic stem cells continues throughout life, and on average, a healthy individual produces more than 500 billion blood cells every day.

Various blood cell lineage differentiates from the HSCs. The two major lineages that HSCs differentiate into are myeloid and lymphoid. The myeloid cells include monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, and megakaryocytes to platelets. Lymphoid cells include T cells, B cells, natural killer cells, and innate lymphoid cells. The myeloid and lymphoid lineages both are involved in dendritic cell formation.

HSCs are generally distributed at a 1:10,000 (one is to ten thousand ratios) in myeloid tissue and constitutes cells with capabilities of long-term and short-term proliferation abilities and are generally committed to multipotent, oligopotent, and unipotent progenitors.

Adult bone marrow is a rich source of hematopoietic stem cells, especially in the pelvis, femur, and sternum. They are also found in umbilical cord blood and, in small numbers, in the peripheral circulation. Hematopoietic stem cells exist in a state of quiescence, or reversible growth arrest. The quiescent HCSs help the cells survive for extended periods of time in the hypoxic bone marrow environment (2016–2017). When provoked by cell death or damage, HSCs exit quiescence and begin actively dividing again. This process is under intricate regulation. The transition from dormancy to propagation and back is regulated by the cell signaling pathway, namely, MEK/ERK pathway and PI3K/AKT/mTOR pathway (Baumgartner et al. 2018).

Dysregulation of these signaling pathways can lead to stem cell exhaustion or the gradual loss of active hematopoietic stem cells in the blood system.

In the early stages of differentiation, HSC gives rise to different subtypes often termed as colony-forming units (CFU). There are seven well-characterized subtypes each giving rise to a definite cell lineage.

- Colony-forming unit-granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM)
- Colony-forming unit-lymphocyte (CFU-L)
- Colony-forming unit-erythrocyte (CFU-E)
- Colony-forming unit-granulocyte-macrophage (CFU-GM)
- Colony-forming unit-megakaryocyte (CFU-Meg)
- Colony-forming unit-basophil (CFU-B)
- Colony-forming unit-eosinophil (CFU-Eos)

2.4.1 Isolation of Hematopoietic Stem Cells

It is difficult to isolate a pure population of hematopoietic stem cells. In general, HSCs are characterized by their small size and low staining with vital dyes such as rhodamine 123 (rhodamine lo) or Hoechst 33342 (side population). The development of Fluorescence-Activated Cell Sorting (FACS) has been crucial characterization and purification of HSCs. FACS is a well-established technique that can accurately recognize and quantify small numbers of cells in large mixed populations of live cells. More importantly, FACS-based cell sorting allows the preparations of HSCs to near 100% purity including rare cell populations. This capability enables the testing of these cells in various assays HSCs can be isolated by flow cytometry where the combination of several different cell surface markers is used to separate the rare HSCs from the surrounding blood cells. HSCs lack expression of mature blood cell markers and are thus called Lin- (Lineage minus). A combination of several positive cell-surface markers along with the lack of expression of lineage markers is used for the detection and isolation of HSCs.

2.4.2 Procedure

The best yield is with fresh samples. It is advisable to free the sample if not processed within 48 h of procurement. The preparation details might need adjustments depending on the specific tissue source. If samples cannot be processed within 48 h, they should be frozen.

Isolation of Human CD34+ Hematopoietic Progenitor Cells

- Samples are diluted in 1:1 in D-PBS without Mg^{2+} or Ca^{2+}.
- Next, 20 ml of Ficoll is poured into a 50-ml tube and layered slowly (tilting the tube and running the cells down the side of the tube), 25 ml of diluted blood or marrow is layered on top.
- The Ficoll-containing tube is then centrifuged at room temperature @1100 g for 20 min.
- Half of the top layer is removed and discarded.

- The "cloudy" interface layer (~10 ml) should be carefully pipetted off and transferred into a clean 50-ml tube. The cells are then washed with 50 mL PBS without Mg^{2+} or Ca^{2+}.
- The cells are next in media with serum or protein (D-PBS or Hanks' solution with 2–6% FBS or HSA).
- The cells are washed twice in D-PBS without Mg^{2+} and Ca^{2+} and centrifuged after each wash.
- RBCs are lysed and removed by resuspending in cold (0.1 M) NH_4Cl solution at 3–4 times the original sample volume followed by incubation on ice for 10 min each time.
- The cells are again washed twice in D-PBS without Mg^{2+} and Ca^{2+} and centrifuged after each wash. The cells are next resuspended in media with serum or protein (D-PBS or Hanks' solution with 2–6% FBS or HSA) at a cell concentration of 10^7/ml.
- The control cells are removed at this point (~10^5 cells/tube) following the technique.
- Unstained cells; irrelevant antibody controls for FITC, phycoerythrin, and Cy5; single-color positive controls for FITC, phycoerythrin, and Cy5.
- The remaining cells are next stained by adding the appropriate antibodies for the chosen procedure. Cells are incubated for 30 min at 4 °C for optimal antibody binding.
- The cells are washed twice and resuspended in Hanks' solution + FBS containing 2 μg/ml propidium iodide (PI) (P4170). Cells are now ready for sorting.
- Appropriate flow cytometer setting are used to sort the cells.

2.4.3 Markers of Hematopoietic Stem Cells

HSC assays, in combination with the ability to purify HSCs, have provided increasingly detailed insight into the cells and the early steps involved in the cell differentiation process. Several marker combinations have been developed that describe human HSCs, including clusters of differentiation markers such as CD34, CD38, Lin, CD90, CD133, and fluorescent substrates for the enzyme, aldehyde dehydrogenase. The use of highly purified human HSCs has been mainly experimental, and clinical use typically employs enrichment for one marker, usually CD34.

2.4.4 Use of Hematopoietic Stem Cells

The clinical use of stem cells holds great promise, although the application of most classes of adult stem cells is either currently untested or is in the earliest phases of clinical testing (Koc et al. 2000); hematopoietic stem cell research has made significant progress. The hematopoietic stem cells are currently the best-studied stem cells backed by more than 50 years of clinical research and investigation. In 2021, more than 1.5 million transplants were performed worldwide. Essentially, bone marrow transplant is a medical treatment that replaces your bone marrow with healthy cells. The replacement cells can either come from your own body or from a donor. A bone marrow transplant is alternatively termed a stem cell transplant or, more specifically, a hematopoietic stem cell transplant. This type of transplantation

can be used to treat certain types of cancer, such as leukemia, myeloma, lymphoma, and other blood and immune system diseases that affect the bone marrow. Thus, currently, the main indications for bone marrow transplantation are either hematopoietic cancers (leukemias and lymphomas) or the use of high-dose chemotherapy for non-hematopoietic malignancies (cancers in other organs). Other indications include diseases that involve genetic or acquired bone marrow failure, such as aplastic anemia, thalassemia sickle cell anemia, and increasingly, autoimmune diseases. The study of HSCs remains active and continues to advance very rapidly. Fueled by new basic research and clinical discoveries, HSCs hold promise for such indications as treating autoimmunity, generating tolerance for solid organ transplants, and directing cancer therapy. One of the major hurdles of HSC transplantation is the availability of matching grants for the diseased host. Currently, one of the major research goals is to achieve the optimal expansion of HSCs. Future developments in genomics and proteomics, as well as in gene therapy, have the potential to widen the horizon for clinical application of hematopoietic stem cells even further.

2.5 Adult Stem Cells

Embryos are not the solitary source of stem cells, rather they are also found in several adult tissues and are important for normal tissue function. Many cells do not live throughout the entire life of the organism and require replenishment. Some cells have a defined life period and eventually die and must be replaced by cell division and differentiation throughout the lifetime of the organism. **Adult stem cells,** also known as somatic stem cells or tissue stem cells, are incompletely differentiated cells residing in many tissues of adult organisms whose normal function is to divide and replace cells that are lost through routine physiological processes. These are termed stem cells and are located in the niche of several tissue regions where cells are continually being lost: skin, gut lining, uterine cervix, bone marrow, and many glands. An important characteristic of a stem cell is that when it divides normally, one daughter cell remains undifferentiated as a stem cell and the other differentiates and is, therefore, less likely to continue dividing. This pool of undifferentiated stem cells is continuously maintained within the tissue niche. Essentially, these processes are tightly coordinated in adult organisms, such that cells lost from specific tissues are replaced by the correct number and type of cells.

Bone marrow is one of the first tissue in the body in which adult stem cells were identified. The porous interior portions of the long bones are a continuous source of pluripotent stem cells throughout an individual's entire life and give rise to both red blood cells, which carry oxygen, and white blood cells, which are part of the immune system. Red blood cells (RBC/erythrocytes) have a life of about 120 days and are required to be produced to replace those that have reached the end of their life span or have been lost through injury. Both the types and numbers of new cells produced are tightly regulated. Deadly diseases like leukemia or myeloma, which are blood cell cancers that develop in the bone marrow, are often a consequence of the disregulation of this process of differentiation. Since the bone marrow contains

pluripotent stem cells, bone marrow transplants can reestablish the pluripotent blood cells and/or the different types of immune cells in individuals lacking them. Transplantation of bone marrow between two individuals requires the exact matching of a set of cell-surface marker proteins; otherwise, the recipient's body may reject the transplanted cells, which can lead to a life-threatening complication called graft-versus-host disease. In some cases, a person's own bone marrow cells can be removed and later transplanted back, for example, after the person's own immune cells have been killed by cancer therapy. This works very well because a person's own cells will not trigger graft-versus-host disease and would be immediately recognized as self.

Adult stem cells are found in small numbers in most adult tissues, such as bone marrow or adipose tissue. Compared with embryonic stem cells, adult stem cells have somewhat limited ability to give rise to various cells of the body. Adult stem cells differ from embryonic stem cells as they are not as undifferentiated as embryonic stem cells; all the same they are still capable of forming many types of cells in addition to the type found in the tissue from which they were derived. However, emerging evidence suggests that adult stem cells may be able to create various types of cells. For instance, bone marrow stem cells may be able to create bone or liver cells. In tissue culture, bone marrow stem cells can develop into other types of cells, including muscle or nerves, given the right set of hormones, and signaling molecules. Transplants of these adult stem cells are being investigated for their future potential to regenerate other types of tissue in addition to blood cells. It appears that, in tissue culture, it may be possible to bring adult stem cells back to full potentiality, that is, the ability to differentiate into any and all kinds of cells. This is not yet known for certain, however.

In the current scientific era, adult stem cells are considered the gold standard for clinical applications and are being repeatedly tested and accepted for a growing number of conditions. The adult stem cells do not have any ethical taboo regarding their isolation, and their practical advantages over pluripotent stem cells have led to many current clinical trials, as well as some therapies approved through all phases of Food and Drug Administration (FDA) testing. There are sufficient peer-reviewed publications claiming successful outcome with adult stem cell therapies. Significant therapeutic benefit in clinical trials and progress toward fully tested and approved treatments have been documented in recent years. Phase I/II trials suggest potential cardiovascular benefits from bone marrow-derived adult stem cells as well as with umbilical cord blood-derived cells. Impressive results have been reported indicating prominent success in treating neurological conditions, including ischemic strokes. Another disease that has shown positive long-term progression-free outcomes is multiple sclerosis and often the disease outcome also included complete remission. Significant benefits in early trials for patients with type I diabetes mellitus and spinal cord injury have been reported. Thus, adult stem cells are starting to be used as vehicles for genetic therapies, such as for epidermolysis bullosa. Several published studies indicate that even in the aged adult human body (age over 60 years) there are multiple tissues that contain multipotent somatic stem cells.

2.5.1 Isolation of Adult (Somatic) Stem Cells

It is being increasingly accepted that stem cells are responsible for the provision of continuous homeostasis of our tissues and organs, which life span is partially determined by the stem cell pool quality, proportional distribution, and local micro-environmental factors. Several types of adult human tissues like bone marrow, adipose tissue, and skin (from different body locations) can serve as potential sources of multipotent somatic stem cells for application in regenerative medicine. Adult stem cells are currently being isolated from many tissues in the body. The methods of isolation and culture are dependent on the source and lineage. Many isolations and purification protocols involve flow cytometry and cell sorting. Positive and negative sorting for cell surface markers can quickly generate enriched populations.

2.5.2 Markers of Adult Stem Cells

Adult stem cells often resemble the mesenchymal stem cell markers and exhibit expression of cell surface markers including CD44, CD90, CD105, CD106, CD166, and Stro-1, lack of the expression of hematopoietic markers, no immunogenic effect, and replacement of damaged tissues. These properties led to the development of progressive methods to isolate and characterization of adult stem cells from various sources for therapeutic applications in regenerative medicine**.**

2.6 Induced Pluripotent Stem Cells

Stem cell research from nonembryonic stem cells has progressed more than embryonic stem cell research. Induced pluripotent stem (iPS) cells, with the same pluripotent characteristics as embryonic stem cells, have replaced embryonic stem cells in many research publications. The iPS cells have distinct advantages compared with embryonic stem cells because they can be sourced from almost any individual or tissue, healthy or diseased, more cheaply and efficiently than embryonic stem cells and without the ethical concerns about their process of creation and isolation. As these are patient-derived cells, there is a high possibility for the production of pluripotent-derived patient-matched cells that can be a therapeutic reintroduction to the patient at a later time. However, great caution is warranted because iPS cells, as with embryonic stem cells, also show genetic instability in culture and may thus show tumorigenic potential**.** The ethical issues regarding embryonic stem cells and the possible limitations of adult stem cells (including difficulties obtaining them from some adult tissues), scientists have sought ways to generate special cells that would exhibit stem-like properties. In 2006, Japanese biologist *Shinya Yamanaka* succeeded in "reprogramming" skin cells from adult mice, returning the cells to a pluripotent state by engineering them to express four transcription activator proteins. The following year, *Yamanaka and colleagues* repeated the accomplishment of using human skin cells. The reprogrammed cells are called induced pluripotent stem cells. Similar to embryonic stem cells, when iPS are exposed to appropriate signals, these induced stem cells will differentiate into a wide range of cell types. This is the

triumph of regenerative medicine with the major benefit of this strategy is that, in theory, any cell type needed could be generated from a patient's own stem cells, which can be easily obtained, and being "self-sourced" will not stimulate any immune reactions. In 2010, *Yamanaka* and British biologist *John Gurdon* were jointly awarded the Nobel Prize in Physiology or Medicine for their discoveries regarding the reversible nature of the differentiation process.

It is yet to be completely explored if induced pluripotential stem cells have a potentiality as broad as embryonic stem cells, as the signals required to specify all cell types have not yet been defined. However, in 2009, mice were born from induced pluripotential stem cells using a technique called tetraploid complementation. These embryos had a low rate of implantation in recipient mothers, and some had physical abnormalities, but some did survive and go on to reproduce, suggesting that the induced pluripotential stem cells were able to give rise to all necessary cell types. Such an experiment would not be considered ethical by humans. Differentiated cells, such as skin cells, can be reprogrammed back into a pluripotent stem cell state. Reprogramming can generally be achieved over several weeks by forced expression of genes that are known to be master regulators of pluripotency. At the end of this reprogramming process, these master regulators would remodel the expression of an entire network of genes. Those associated with the pluripotent state, essentially reversing the developmental process, will replace features of differentiated cells.

2.6.1 Isolation of Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are essentially the somatic cells that have been reprogrammed to become pluripotent. Thus, any somatic cell could be essentially and theoretically reprogrammed. Initially, somatic cell nuclear transfer (SCNT) technique was tested out for understanding the process of reprogramming. The first somatic cell nuclear transfer (SCNT) experiments were successful in demonstrating that somatic cells possess a full genome and opened the door to the reprogramming of somatic cells to pluripotent state. Although this technique played a crucial role in our understanding of pluripotency and remains one of the most efficient methods available to reprogram somatic cells, SCNT is not an ideal strategy for producing pluripotent cells because it requires the use of an unfertilized egg. The isolation and manipulation of unfertilized eggs can be only utilized in the setting of in vitro fertilization, but the scarcity of eggs in the research setting prohibits large-scale use of SCNT. Thus, new technology was necessary for the ethical and efficient generation of pluripotent stem cells. For many stem cells like blood cells, reprogramming have been relatively easy and straightforward while others like the cardiac cells are more technically challenging. At first, iPSCs were generated by the introduction of the transcription factors Oct3/4, Sox2, Klf4, and c-Myc in cells maintained in culture conditions. Other investigators have since used various combinations of these transcription factors. In 2006, the *Yamanaka group* overcame this ethical controversy when they described a protocol whereby somatic cells could be dedifferentiated into a pluripotent state following the transduction of a four previously mentioned transcription factor cocktail. Following this initial study, numerous

groups have described protocols to generate induced pluripotent stem cells (iPSCs). These protocols have simplified the reprogramming strategy by employing polycistronic reprogramming cassettes and flanking such polycistronic cassettes with loxP or piggyBac recognition sequences.

The introduction of relevant transcription factors cloned into plasmid expression vectors was utilized at very early-stage experiments, but the efficiency was low, and occasionally expression plasmids can integrate into genomic DNA. This was followed by the introduction of transcription factors cloned into retroviral and lentiviral vectors and since has been the most widely adopted technique for generation of the iPSCs. There were several concerns raised regarding the gene silencing epigenetic effects of the viral vectors. A major consideration for using retroviruses to generate iPSCs is that the viruses integrate into the host DNA. Depending on the integration site, integration can have deleterious effects on the cells, altering gene expression and increasing the risk of tumor formation. *Stadfield and associates* in 2008 used adenovirus as an alternative, and Sendai virus, an RNA virus, was contemporarily used by *Seki and colleagues*, as vectors to transduce transcription factors since these do not integrate into the genomic DNA of the cell. Furthermore, the retro/lenti-mediated introduction of transcription factors is often very inefficient. A more efficient method was found to be the combination of lentiviruses and microRNAs (miRNAs) to reprogram cells were reported by *Anokye-Danso and accomplices* in 2012. These small RNAs bind to mRNA and either inhibit translation or cause degradation of transcripts. miRNA clusters, including miR-290-295 and miR-302-367, have been shown to enhance reprogramming of somatic cells into iPSCs.

Another approach avoiding virus-mediated transduction of transcription factors was the use of miRNA mimics and the viral vector to enhance expression of transcription factors. miRNA mimics are double-stranded modified RNAs that mimic mature miRNAs (fully processed cellular miRNAs). miRNA mimics do not require a vector and are capable of being transfected directly into cells. *Judson and accomplices* in 2009 have shown that the combined use of transcription factors and miRNA mimics produces more homogeneous iPSC clones. An advantage of using miRNA mimics with transcription factors is that the transcription factor c-myc, an oncogene, is not required.

Apart from viral vectors and miRNA mimics, several chemical compounds that modulate enzymes controlling epigenetic modifications have been evaluated for increasing the efficiency of transduction by transcription factors. DNA methyltransferase and histone deacetylase (HDAC) inhibitors were shown to potentiate the efficacy of transduction. It has been reported that HDAC inhibitor valproic acid was the most effective, increasing reprogramming efficiency by 100-fold. With the miRNA mimics, the use of valproic acid eliminated the need to transduce the oncogene c-myc. A number of other inhibitors of kinases, such as the glycogen synthase kinase-3 (GSK3) inhibitor CHIR99021 and the MEK inhibitor PD0325901, and other enzymes that are in pathways involved in pluripotent induction also exhibited an enhanced efficiency in reprogramming.

2.6.2 Markers of Induced Pluripotent Stem Cells

Undifferentiated induced pluripotent stem (iPS) and embryonic stem (ES) cells share many common markers that can be functionally characterized by the ability to differentiate into cells of the three germ layers. In addition to the ability to give rise to all cell types, a number of molecular markers have been identified to verify the pluripotent status of stem cells. For example, human pluripotent stem cells express the cell surface proteins SSEA-4 and alkaline phosphatase and the transcription factors Oct-3/4 and Nanog. The ability to verify stem cell pluripotency using established markers at the start of an experiment helps ensure that downstream stem cell proliferation and differentiation studies are conducted on high-quality, undifferentiated starting cell populations.

3 Stem Cell Identification

CD34 Positive marker is expressed in all human bone marrow (BM) progenitor cells.

HSCs can be isolated using flow cytometry based on surface marker expression. There are several criteria that have to be taken into consideration when the isolation of HSCs has to be performed. For most purposes, multipotent hematopoietic progenitors should be CD34+/CD38-/CD45RA-/CD71- in the expression of surface markers. CD133+, CD90+ (Thy-1), ALDH+, and Sca-1+ are also expressed in progenitor stem cells and are considered positive or expressing markers. The absence of expression of CD2-, CD3-, CD19-CD41-, CD16-, CD14-, and CD15- is also a hallmark of hematopoietic progenitor stem cells and are considered negative markers thus should be non-expressing for bone marrow progenitors and included in the list of mature blood lineage (Lin-) markers.

4 Stem Cell Expansions

4.1 Expansion of Human CD34+ Hematopoietic Progenitor Cells

- Cell culture media is prepared by combining the Basal Medium and the Supplement Mix of the PromoCell HPC Expansion Medium DXF (Sigma C-28021) according to the manufacturer's instructions. An appropriate volume of PromoCell Cytokine Mix E (Sigma C-39890/C-39891) is carefully added to generate the completely supplemented Expansion Medium. The complete and supplemented media should be pre-equilibrated at 37 °C and 5% CO_2 for 30 min by placing the media in the 37 °C incubator.
- To the pre-equilibrated medium, the freshly isolated HPCs plated at a density of 5000 cells/ml. When using cryopreserved HPCs, thawing of the cells is necessary for 2 min in a 37 °C water bath. Post-thawing, the HPCs are transferred into the

pre-equilibrated medium at a density of 5000 cells per ml. It is recommended that at least 9 ml of medium is added per vial of cryopreserved cells.

- The cells are incubated for 96 h at 37 °C and 5% CO_2. If necessary, partial medium change can be performed. Essentially, cells are removed from the incubator and pipetted up and down several times to prepare a single cell suspension. The entire content of the tissue culture plate is then transferred into a 50 ml tube. The cells were next centrifuged for 10 min at 240 x g. Then, carefully aspirate the upper two-thirds of the medium. Gently resuspend the cells in the remaining third of the medium and replenish to the original volume with fresh cytokine-supplemented HPC Expansion Medium DXF.
- Cells are to incubate for another 6–8 days to allow sufficient expansion. About two-thirds of the medium is replaced as described above every 72.
- Cells are next collected from the tissue culture plate containing the expanded HPCs. It is better to pipette up and down the culture media several times in order to release loosely attached cells and to achieve a single-cell suspension. The HPCs cells are finally harvested by centrifugation at 240 x g for 10 min following which the supernatant is discarded.
- The cells are finally resuspended in Promo Cell HPCs Expansion Medium DXF (Sigma C-28021) and the cell number is determined using a hemocytometer and trypan blue staining. The HPCs are then ready for a new experiment.

5 Stem Cell Differentiation

HSCs have a few unique properties, the combination of which defines them as such. The core properties are the ability to choose between self-renewal or differentiation which is essentially embarking on becoming a mature hematopoietic cell. HSCs migrate in a regulated fashion and the population is significantly controlled by apoptosis (programmed cell death). The balance between these activities determines the number of stem cells that are present in the body. Hematopoietic stem cells can be differentiated in vitro by using relevant cytokines that would promote the differentiation into a specific lineage. Colony-stimulating cytokines and other cellular components signal via their cognate receptors to regulate hematopoiesis. These cytokines are termed hematopoietic cytokines.

Hematopoietic cells are under the tight control of a group of hematopoietic cytokines. Each cytokine exerts multiple actions mediated by receptors whose cytoplasmic domains contain specialized regions initiating the various responses that include survival, proliferation, differentiation commitment, maturation, and functional activation. It has been postulated that cytokines, signaling via their cognate receptors, may play an instructive role in lineage specification in hematopoiesis. Individual cytokines can be lineage-specific or can regulate cells in multiple lineages. The simultaneous action of several cytokines is often required for proliferative responses. Example of such cell types includes progenitors of megakaryocyte and their progenitor stem cells. These cytokines also control basal and emergency hematopoietic cell proliferation. The three major cytokines, erythropoietin,

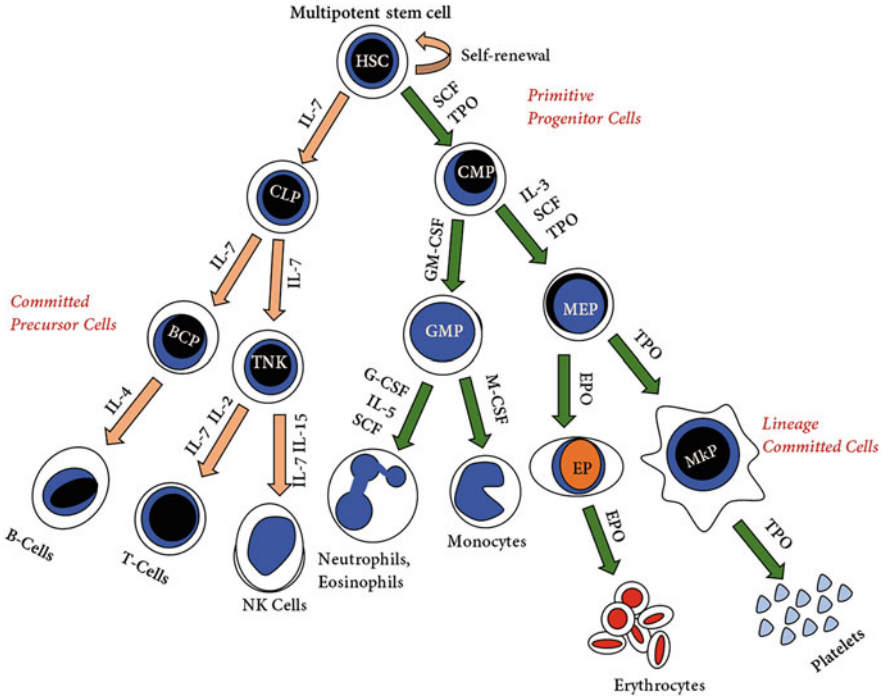

Fig. 3 A schematic representation of the lineage adopted by hematopoietic stem cells. Essentially, they are capable of both self-renewal and/or differentiation when the signal is received. The CLP (common lymphoid progenitor) and CMP (common myeloid progenitor) are considered to be primitive progenitor cells whereas BCP (B cell precursor), TNK (T and natural killer cell precursor), GMP (granulocyte-macrophage progenitor), MEP (megakaryocyte erythroid progenitor), and BCP (B cell progenitor) are partially differentiated progenitor cells. Different cytokines promote differentiation and are controlled by a cascade of the signaling pathway

granulocyte colony-stimulating factor, and granulocyte-macrophage colonystimulating factor, are now been used in routine clinical practice to stimulate cell production, and thus are routinely employed in patient management.

Hematopoietic cytokines are a large family of extracellular ligands that stimulate hematopoietic cells to differentiate into eight principal types of blood cells. Hematopoiesis requires the synergistic action of several cytokines that act both as positive and negative regulators functioning within a complex network of actions. Some cytokines have very narrow lineage while many others have rather broad and overlapping specificity ranges. Cytokines whose predominant action appears to be the stimulation or regulation of hematopoietic cells include GM-CSF, G-CSF, M-CSF, interleukins, EPO, and TPO. There are several other cytokines that exert profound effects on the formation and maturation of hematopoietic cells, which include stem cell factor (SCF), flt-3/flk-2 ligand (FL), and leukemia inhibitory factor (LIF). Other cytokines or ligands such as jagged-1, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) also play significant roles in modulating hematopoiesis (Fig. 3).

5.1 Cancer Stem Cells

Cancer is characterized as a monoclonal disease originating from a single apparently transformed cell. It is accepted that a single cell undergoes transformation and then escapes immune surveillance and continues to proliferate to form a colony of cancerous cells. Despite great advances in our understanding of tumor initiation and progression, the identity of the "cell of origin" of cancer remains elusive. It is now established that these cells have self-renewal properties analogous to stem cells. The cancer stem cells (CSC) constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. These cancer stem cells have the capacity to both divide and expand the cancer stem cell pool and to differentiate into the heterogeneous non-tumorigenic cancer cell types that in most cases appear to constitute the bulk of the cancer cells within the tumor (Clarke et al. 2006). Solid tumors are an enormous cancer burden and a major therapeutic challenge. The cancer stem cells (CSC) provide important cues to cellular mechanisms that may explain the probable reasons for the chemotherapy refractoriness and dormant behavior often observed for many of these neoplastic growths. There is increasing evidence that diverse solid tumors are hierarchically organized and sustained by a distinct subpopulation of CSCs (Visvader and Lindeman 2008).

An alternative theory for the origin of CSCs suggests that they arise from normal somatic cells that acquire stem-like characteristics and malignant behavior through genetic and/or heterotypic alterations. For example, cancer cells gain stem-like characteristics through epithelial-mesenchymal transition (EMT). If cancer stem cells are relatively refractory to therapies that have been developed to eradicate the rapidly dividing cells within the tumor that constitute the majority of the non-stem cell component of tumors, then they are unlikely to be curative and relapses would be expected. If correct, the cancer stem cell hypothesis would require that we rethink the way we diagnose and treat tumors, as our objective would have to turn from eliminating the bulk of rapidly dividing but terminally differentiated components of the tumor and refocused on the minority stem cell population that fuels tumor growth (Clarke et al. 2006). Several cell surface markers such as CD44, CD24, and CD133 are often used to identify and enrich CSCs. A network comprising microRNAs along with Wnt/β-catenin, Notch, and Hedgehog signaling pathways regulates the CSC properties. Since CSCs are resistant to different types of chemotherapies and radiation treatments, it is hypothesized that CSCs play a crucial role in cancer metastasis. CSCs are believed to be an important target for novel anticancer drug discovery (Yu et al. 2012). Initially, CSCs were believed to represent a small fraction of the total cell population in a solid tumor; however, it has been claimed that as many as 25% of cancer cells may have the properties of CSCs. An alternative theory for the origin of CSCs suggests that they arise from normal somatic cells which acquire stem-like characteristics and malignant behavior through genetic and/or heterotypic alterations. For example, cancer cells gain stem-like characteristics through epithelial-mesenchymal transition (EMT). The induction of EMT in immortalized human mammary epithelial cells (HMLEs) resulted in the acquisition of

mesenchymal traits and expression of stem-cell markers, which are similar to those stem cell-like cells isolated from HMLE (Yu et al. 2012).

It has been reported that freshly isolated patient-derived $CD133^+$ CSC were highly tumorigenic. When as few as 500 cells were implanted in athymic mice, orthotopic tumor formation was observed. In contrast, as many as 10^6 CD133-tumor cells did not result in any tumor formation. Most importantly, it was shown that CSC does not represent a homogeneous population of tumor-initiating cells. Instead, it was defined that a subpopulation of migrating CSC that were characterized by expression of the CXCR4 receptor and are critically involved in tumor metastasis. Indeed, the elimination of this subpopulation of CSC virtually abrogated the metastatic activity of CSC. It is thus confirmed that cancer stem cells arise from normal stem cells possibly by a series of mutations. Some evidence also indicates that CSC may also arise from mutations of the progenitor cells. Cancer stem cells are capable of undergoing symmetric and asymmetric divisions, which consequently leads to the clonal expansion of the tumor cells. This leads to the production of a population of the progeny of differentiated cells that constitute a major portion of the tumor cell mass. CD133 is universally expressed in many cell types that include cells of normal tissue as well as hematopoietic stem cells. As demonstrated for hematopoietic and endothelial progenitors, CD133 is also expressed on early progenitors but usually is no longer detectable upon differentiation (Singh et al. 2004). In tumor cells, CD133 has been used for the identification of a subpopulation of highly tumorigenic cells as demonstrated for neural cancers and, more recently, also for cancers of the colon (Singh et al. 2004). The expression of CD133 is no longer expressed on undifferentiated tumor cells once they undergo differentiation. Thus, the hypothesis is that a subpopulation of CD133+ cells that bear self-renewal capacity as they can be clonally expanded are exclusively tumorigenic and is able to differentiate into CD133 positive tumor cell clone.

CSCs were believed to represent a small fraction of the total cell population in a solid tumor; however, it has been claimed that as many as 25% of cancer cells may have the properties of CSCs. There are several different explanations regarding the origin of CSCs. As discussed earlier, one of the strongest theories is that genetic mutation or environmental alterations is the primary cause that induces the transformation of CSCs from normal stem/progenitor cells and then develops the ability to generate tumors. Some CSCs exhibit similarities to normal stem/progenitor cells in cellular property, phenotype, function, and even cell surface markers. It is becoming evident that a cancer treatment that fails to eliminate cancer stem cells may allow the regrowth of the tumor. In cases of disease relapse post-eradication of the tumor tissue by chemotherapy indicates that cancer stem cells remain viable after the destruction of the tumor tissue. Recent studies have indicated that CSCs have the intrinsic characteristics to become resistant to cancer therapies. Cancer research attempts to identify mechanisms that drive drug resistance. Similar to normal stem cells, the CSCs confer the abilities of self-renewal and can serve as a core reservoir of cells, thus making the disease of cancer incurable. The overexpression of stem cell-specific transcription factors may contribute to the pathological self-renewal characteristics of cancer stem cells while the surface molecules mediate interactions between cells

and their microenvironment. Several other studies implicate that there are several other stemness-related markers and proliferative pathways that may promote cancer development and maintenance. Therapeutic strategies that specifically target cancer stem cells are urgent and should eradicate tumors more effectively than current treatments and reduce the risk of relapse and metastasis.

5.1.1 Cancer Stem Cell Isolation

The existence of cancer stem cells (CSC) has been documented for the past two decades. However, the studies related to CSCs pave a number of issues due to rare cell populations and difficulties in their isolation which for the most part due to their similarities to common stem cell markers. CSCs are frequently found in the core of solid tumors and their microenvironment plays an important role in tumor maintenance, renewal, division, and development. Thus, in vivo tracking is an important technique for functional studies of cancer stem cells. CSCs are commonly resistant to chemotherapy and can introduce dormancy in tumor cells for extended periods of time. Therefore, proper detection, isolation, and characterization of the undifferentiated stem cells residing within solid tumors are the potential markers for the development of cancer-targeted therapies in recent years. Remarkable similarities and identities between normal and cancer stem cells make it a challenge to delineate specific methods or markers to distinguish them individually and identify markers that can be therapeutically challenged. Tumors generally originate from the transformation/alterations of normal stem cells; hence some markers continue to be common to both types of stem cells. In a similar vein, the signaling pathways may regulate self-renewal in stem cells and cancer cells, and thus cancer cells may include "cancer stem cells" – a rare type of cells with indefinite potential for self-renewal that induces tumorigenesis. Due to ethical reasons, it has been difficult to isolate cancer stem cells from human patients. During primary resection of the neoplastic tumor, the isolation must be organized with the laboratory team which is often difficult in hospital settings. Most of our knowledge regarding the characteristics and salient properties of CSC has been gathered from mouse xenograft studies.

Mouse Xenografts CSC Isolation (Adopted: Dobbin and Landen 2013)

CSCs constitute a small subset of the cancer cells in a heterogeneous tumor. As such, it is necessary to dissociate a tumor sample into a single-cell suspension to be able to isolate CSCs from the rest of cancer. Although some studies have taken advantage of marker-positive and -negative populations identified within cell lines, it is generally believed that for a population to be considered a CSC, increased tumorigenicity from a patient-derived tumor and the capacity to reproduce the original tumor are required. This adds additional challenges, as dissociation of a solid tumor into a single-cell population is traumatic to cells and time-consuming. In this method, it will be described how to mechanically dissociate a tumor tissue sample that comes either from a human patient or a mouse xenograft.

5.1.2 Materials

- Tumor sample preferred at least 1 cm of viable tissue
- Serum-free RMPI cell culture media (or preferred media)
- 10 cm Petri dish
- Scalpel handle and #22 blade
- 50 ml conical tube
- Tissue forceps
- 70 μm sterile mesh filter
- 10 ml, 5 ml, and 1 ml pipette tips
- 16-gauge needles and 3–5 ml syringes
- Hemocytometer, trypan blue

5.1.3 Protocol

- Start processing the tumor without delay to maximize viability. Ideally, post-tumor resection, the tumor should immediately be subjected to processing which should begin within 30 min of removal. If this can be achieved, then the tumor tissue can be implanted in mice or culture plates in another 30–60 min. Sometimes, this is not possible due to the unavailability of the personnel and lateness in pathologic review. Prior collaboration should be initiated between the surgeon and the pathologist to prevent any compromise in patient care and early retrieval of the excised tissues. On the other hand, mouse tumors can generally be obtained more quickly, as they are resected immediately after sacrifice, can be identified grossly, and processing can begin immediately.
- Patient or mouse-derived tissue specimens should be placed in a 10-cm dish with 1 ml cold media. The dish must be kept on ice or an ice pack during dissection.
- The solid mass has to be next disintegrated/minced using forceps to hold the specimen firmly and use the back of a number 22 scalpel blade to scrape the specimen downward, and away, such that cells are loosened from the tumor mass into the 10-cm dish. During this process, strands of connective tissue can be isolated, and should ideally be removed from the cell collection dish.
- The process of scraping should be continued till the specimen is exhausted and can no longer be held with the forceps and a large "slurry" population has been collected in the 10-cm dish.
- The cell slurry is further disintegrated first with a 10 ml serological pipette, then to a 5 ml pipette, then a 1 ml pipette, until the slurry passes easily in and out easily.
- A 16-gauge needle on a 3- or 5-ml syringe is then used for complete mechanical dissociation.
- At this point, the cells should be ready for downstream experiments such as injection into mice, or for tissue culture. If a single-cell suspension is required for performing flow cytometric analysis, the next few steps are necessary.
- The cell suspension has to be next filtered using a 70-μm filter placed on top of a 50 ml conical tube. The cell suspension should be poured slowly onto the filter and allow the cell suspension to accumulate in the collection tube. Clogging can happen quickly if the suspension has largely unbroken tissue mass; thus, the prior

steps are extremely important. In such eventuality, the suspension should be passed through 200 μm and 100 μm filters, and then only through 70 μm filters.
- After collection of the entire cell suspension, 50 μl of the cells are taken out and used for counting and assessment of the viability. The remaining cell sample is centrifuged at 3000 rpm (1500 × g) for 10 min at 4 °C.
- While samples are centrifuging, the viability and cell number in the suspension are determined by using a hemacytometer and trypan blue staining.
- Media must be aspirated off followed by resuspension of the cellular pellet in cell culture media or PBS as required for downstream applications. The appropriate volume to be used is dependent on the applications to be utilized at the later time point.

5.1.4 Alternate Protocol Mouse Xenografts CSC Isolation: Chemical Dissociation (Adopted: Dobbin and Landen 2013)

The decision to use a mechanical-based dissociation or a chemical-based dissociation is primarily a personal preference and is also guided by the density of the tumor. Avoiding chemical dissociation is always a better preference, as this can be corrosive, and has been reported to reduce viability when adding this method. Some tumor types are more amenable to mechanical dissociation with high viability while others tend to be more appropriate for chemical digestion. This varies not only with tumor type, but the site of collection and patient heterogeneity. Therefore, the decision to add chemical dissociation is often a case-by-case basis, depending on the success of mechanical dissociation. If the tumor is especially dense, starting with chemical dissociation may provide maximal yield and viability. Alternatively, a combination can be used, whereby cells are first mechanically dissociated using the above protocol, followed by chemical digestion of firm residual tumor that was not released by this method. The principles of chemical dissociation are using an enzyme to digest the physical bonds between the tumor cells and the extracellular matrix.

5.1.5 Materials

- Enzymatic digestion solution (0.25% Trypsin/EDTA). The ideal digestion solution will depend on cell type to maximize viability. Commonly used digestion solutions include hyaluronidase at 0.05 mg/ml and collagenase at 0.5 mg/ml.
- Tumor fragments
- RPMI-1640 medium with 10% fetal bovine serum
- 1X PBS
- Trypan blue
- 70 μm sterile mesh filter
- 10 cm Petri dish
- No. 22 scalpel blade and blade handle
- 50 ml conical tube
- Tissue forceps
- 10 ml, 5 ml, and 1 ml serological pipette

5.1.6 Protocol

- After extraction of the tumor from the mouse or the patient, place the tumor in 10 ml of 1X PBS in a 50 ml conical tube and store the sample on ice until ready to begin dissociation
- Add 10 ml, 0.25% trypsin/EDTA with or without hyaluronidase and collagenase to the 50 ml conical tube. If the sample is small, can use 5 ml PBS and 5 ml chemical digestion solution in a 10 ml conical tube.
- Begin to dissociate the sample using the No. 22 scalpel blade and cut up the tumor sample in a chopping motion taking care not to crush the sample.
- Place the sample in the conical tube and incubate at 37 °C for 20 min.
- Further dissociate the tumor sample by pipetting up and down the solution using a 10 ml serologic pipette until it passes freely, then a 5 ml and 1 ml pipette.
- Neutralize the trypsin-cell solution with 20 ml, RPMI-1640 medium with 10% FBS (or 10 ml in a 15 ml conical tube).
- Using the 5-ml pipette, pass the cell suspension through a 70 μm sterile mesh filter placed over a new 50 ml conical tube to generate a single-cell suspension
- Once the entire cell suspension has been collected, collect 50 μl to use for counting and assessment of the viability, and centrifuge the remaining sample at 3000 rpm (1500 × g) for 10 min at 4 °C.
- While samples are centrifuging, determine the number and viability of cells in the suspension using a hemocytometer and trypan blue exclusion.
- Aspirate off media and resuspend the cellular pellet in cell culture media or PBS as desired for downstream applications, in the appropriate volume needed for these applications, based on cell density calculated by trypan blue exclusion.

5.1.7 Cancer Stem Cell Markers

Cancer stem cells have been appreciated as a small subpopulation of cells within tumors with capabilities of self-renewal, differentiation, and tumorigenicity when transplanted into an animal host. CSCs similar to adult and embryonic stem cells may express markers that are not expressed in normal somatic cells and are thus marked to contribute toward a "stemness" phenotype. Thus, stemness-related CSC surface markers that are highly expressed in stem cells are also expressed in human cancers include TRA-1-60, SSEA-1, EpCam, ALDH1A1, Lgr5, CD13, CD19, CD20, CD24, CD26, CD27, CD34, CD38, CD44, CD45, CD47, CD49f, CD66c, CD90, CD166, TNFRSF16, CD105, CD133, CD117/c-kit, CD138, CD151, and CD166. Among these, CD44 and CD133 are the most widely used markers in CSC research and are therapeutic targets in cancers. Of many cell surface markers, CD44, CD24, and CD133 are often used to identify and enrich CSCs within the niche of the tumor microenvironment. Several stemness-related markers have recently been identified and in fact, they are neither transcription factors nor cell surface proteins and include ALDH, Bmi-1, Nestin, Musashi-1, TIM-3, and CXCR. Furthermore, a regulatory network consisting of microRNAs and Wnt/β-catenin, Notch, and Hedgehog signaling pathways have also been documented to control the CSC properties. Emerging scientific evidence (Yu et al. 2012) has strengthened the clinical relevance of CSCs.

5.1.8 Stem Cell Therapy and Remaining Challenges

Although there are some successes documented so far, there are several major challenges that must be addressed before stem cells can be used as cell therapies to treat a wider range of diseases.

First and foremost is the identification of an abundant source of stem cells. Identifying, isolating, and growing the right kind of stem cell with proper stimulation and differentiation, particularly in the case of rare adult stem cells, is an extremely painstaking and difficult process. Pluripotent stem cells, such as embryonic stem cells, can be grown indefinitely in the lab and have the advantage of having the potential to become any cell in the body, but these processes are again very complex and have been tightly regulated. Induced pluripotent stem cells, while promising, are also limited by these conditions. In both cases, considerable work remains to be done to ensure that these cells can be isolated and used safely and routinely within a clinical environment.

Second, as with organ transplants, a close match between the donor tissue and the recipient is crucial; the more precisely the tissue matches the recipient, the lower the risk of rejection. This would ensure that the recipient could avoid the lifelong use of immunosuppressants. The discovery of iPS cells has opened the door to developing patient-specific pluripotent stem cell lines that could later be developed into a needed cell type without the problems of rejection and immunosuppression that occur from transplants from unrelated donors.

Third, a system for delivering the cells to the right part of the body must be developed. Once in the right location, the new cells must then be encouraged to integrate and function together with the body's other cells. The immune reaction post-transplantation is another aspect that requires careful vigilance. Thus, a comprehensive management plan has to be developed to successfully include stem cell therapy as a routine clinical treatment.

Although many signs of progress have been made in delineating the molecular basis of cancer, the progress in cancer detection and treatment, mortality is still high and there is no definitive cure despite great improvements made in various therapies. Stem cell-based regenerative therapies have raised hopes for novel therapeutic approaches. Stem cells are cells that can perpetuate themselves through their ability of self-renewal and to generate mature cells of a particular tissue through differentiation.

6 Conclusions

Stem cells are the foundation for every organ and tissue in the human body. There are many different types of stem cells originating from different tissues within the body and are generated at different stages of growth and development. The embryonic stem cells that exist only at the earliest stages of development are important as various types of tissue-specific stem cells that appear during embryonic development and continue to remain in human tissues throughout life. Stem cells occur in many different somatic tissues and are important participants in cellular physiology. Cell

populations derived from stem cells are organized in an ordered fashion, with the stem cell placed right at the apex of the developmental pathway. Stem cells have three distinctive properties: self-renewal, the ability to develop into multiple lineages, and the capacity to proliferate extensively. The combination of these three properties makes stem cells unique and different from matured differentiated somatic cells.

The specialized class of somatic stem cells or adult stem cells supports and serves as an internal repair system that generates replacements for cells that are lost through normal wear and tear injury, or disease throughout the life of an organism. Populations of adult stem cells have been identified in many organs and tissues and are generally associated with specific anatomical locations. These stem cells may remain quiescent (nondividing) for long periods until activated by a normal need for more cells to maintain and repair tissues.

Every day, new scientific evidence indicates that stem cells are present in many more tissues and organs than once thought of. These stem cells are capable of developing into more kinds of cells than previously perceived. Efforts are being invested to harness stem cells and to take advantage of self-renewal capabilities, with the goal of devising new and more effective treatments for several diseases. The future of stem cell biology is still unclear but it is definite that there are vital research questions to be answered and that those answers will hold great promise for the future of mankind.

To effectively administer stem cell therapies to diseases, stem cell researchers manipulated stem cells so that the cells have the necessary characteristics for successful differentiation, transplantation, and engraftment. A stupendous degree of research is in progress to develop procedures to utilize stem cell populations for the betterment of human health and include procedures like the induction and development of blood vessels (vascularization) for the regeneration and repair of solid tissues. The attribute of self-renewal is especially notable because its subversion is highly relevant to oncogenesis and malignancy. Increased self-renewal capacity, along with the inherent growth potential of the stem cells, might also be the primary cause of the development of aberrant cell growth which can be alternatively considered a neoplastic phenotype. Stem cell therapies although having tremendous promise is still in their infancy. It still assures hope for the future.

7 Cross-References

- ▶ Common Reagents and Medium for Mammalian Cell Culture
- ▶ Culture of Neuron and Glia Cells
- ▶ Isolation and Primary Culture of Various Mammalian Cells
- ▶ Mammalian Cells, Tissues and Organ Culture: Applications

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Mammalian Cell Culture in Three Dimensions: Basic Guidelines

Parth Malik and Tapan Kumar Mukherjee

Contents

Abstract

Mammalian cell culture has technologically advanced from being conducted in two- to three-dimensional culture vessels (**2D** and **3D,** respectively). The architecture of optimum 3D mammalian cell culture vessels closely resembles the **in vivo physiological environment** of mammalian cells/tissues/organs. For 3D culture, five different platforms, viz. **scaffolds, scaffold-free spheroids, gels, bioreactors,** and **microchips,** are used. These platforms are constituted of **collagen, fibronectin, gelatin, laminin, vitronectin,** or other synthetic materials such as **agarose,** all of which resemble mammalian extracellular matrix **(ECM).** A variety of techniques, such as **3D printing, particulate leaching, electro-spinning,** etc. are used to prepare various 3D platforms. In today's scientific world,

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_13-1

mammalian cell culture in 3D has considerable utility, not only in the **research laboratory to understand the physiology of various cells** but also in **clinical (such as in cancer biology), regenerative medicine, and organ to organism developments.**

Keywords

3D cell culture · Platforms for 3D cell culture · Scaffolds · Scaffold-free spheroids · Gels (Matrigel/Hydrogel) · Bioreactors · Microchips · Organ-on-chip

1 Introduction

This chapter focuses on mammalian cell culture techniques primed using three-dimensional (3D) platforms. Three-dimensional cell culture could be defined as the culture of living cells within the microfabricated devices or platforms having a 3D structure that mimics tissue and organ-specific microarchitectures. The specific architecture used in a 3D cell culture resembles the normal anatomical and physiological conditions of a mammalian body. The main purpose of 3D cell culture is to mimic the in vivo natural cellular response and therefore reduce the sacrifice of animals for experiments. Providing 3D architecture in laboratory conditions is a deemed fit requirement for multipurpose large-scale mammalian cell production, such as the production of recombinant proteins, enzymes, antibodies, therapeutic entities, etc. (Pampaloni et al. 2007).

In comparison, mammalian cell growth in two dimensions (2D) involves their culturing on flat cell culture containers/vessels devoid of replicating in vivo physiological conditions. Five different platforms, namely, **scaffolds, scaffold-free spheroids, gels, bioreactors,** and **microchips,** are used in 3D cell culture. A scaffold is a highly porous polymeric biomaterial structure (**matrix**) that acts as a 3D platform to grow the cells or regenerate the tissues. Thus, scaffolds are also called **3D matrices**. Most commonly used scaffolds are made up of either natural extracellular matrix (**ECM**) materials such as **collagen, fibronectin, gelatin,** and **laminin, or vitronectin** synthetic materials such as **agarose**. Scaffolds are manufactured using a variety of techniques, such as **3D printing, particulate leaching,** and **electrospinning**. Scaffolds find considerable utility, not only on the **laboratory scale** but also in **clinical** and **regenerative medicine** (Chitcholtan et al. 2012).

Spheroids are self-assembled spherical clusters of cell colonies. These can be as large as 500–600 μm in diameter. Various methods are used to form spheroids, such as **pellet culture, spinner culture, hanging drop, liquid overlay, rotary wall vessels, external force, cell sheets, microfluidics, and micromolded nonadhesive hydrogel.** Spheroid culture has widespread applications in **bioprocessing** and **organ culture technology**. **Gels for 3D culture give a soft tissue with requisite stiffness and ECM mimicking nature**. Gels are made up of either natural grade materials such as **collagen** or**iginators** or synthetic materials, such as **polyethylene glycol (PEG)-based hydrogel.** One important 3D-constituent gel material is **matrigel.** It is

a reconstituted basement membrane extract that is derived from the Engelbreth-Holm- Swarm (**EHS**) mouse sarcoma. At present, **bioreactors** are mainly used for large-scale culture of mammalian cells for various industrial purposes. Now, these bioreactors are adapted to 3D culture by the scaffolds. Additionally, an "**organ on a chip"** or **microsystem or microchips** are utilized for the 3D culture of mammalian cells. Within the microfabricated 3D devices, each of these techniques has its advantage and disadvantage. Toward the end, this chapter enhances our knowledge about the mammalian cell culture in a 3D setting (Lee et al. 2008).

2 Advantages and Limitations of Three-Dimensional Over Two-Dimensional Cell Culture Approach

The 3D cell culture techniques have the following advantages over the 2D approach*:*

- Three-dimensional cultured cells exhibit features that are pretty much similar to cells present inside the body, maintaining the 3D anatomy and physiology architecture of a mammalian body. The phenotypic heterogeneity of the cultured cells is maintained in 3D culture. Experimentally it is confirmed that as compared to 2D cultured cells, 3D cultured cells are characterized by a higher level of tight junctions, extracellular matrix (ECM) synthesis, and mucin secretion. Thus, cell-to-cell contact and cell–to-matrix contact are better developed in 3D cultured cells, and therefore proliferation, differentiation, and other physiological actions of the cells are close to those of the in vivo conditions. In contrast, the 2D cultured cells are grown in flat cell culture containers/vessels and are, thus, unable to exhibit characteristic features of cells present in vivo. Missing specific architecture and shape of the cell culture containers (plates/flasks) is the major reason for this failure to exhibit characteristics in vivo features.
- In 3D cell culture, cells are attached by natural cell-to-cell adhesion, because of the ECM-like characteristics of the extracellular space. In contrast, the 2D cultured cells cannot attach properly due to missing stroma and specific culture-container architecture.
- Compared to 2D cultured cells, the gap junctions are widespread in a 3D environment. These gap junctions help in **cell-to-cell cross-talk** via the exchange of ions, small molecules, and electrical currents.
- Three-dimensional cultured cells demonstrate the capacity of **differentiation**, a feature missing in two-dimensional cultured cells. Artificial organ formation can never be possible in a 2D cell culture environment. Stem cells, both embryonic and adult, promise to revolutionize futuristic medicinal practices. Most importantly, the signaling mechanisms, necessary to control the differentiation of stem cells into tissues of interest, remain to be elucidated, owing to which much of the present stem cell research is focused on this goal. Stem cell culture in 3D is one of the most important areas of present-day research.

- The 3D cultured human tissues are used to monitor for the infection with infectious microorganisms and the mechanism of disease development by this infectious microorganism in the 3D cultured human cells. These cells are further utilized to discover the mechanism of action of various antimicrobial agents or drugs, the development of drug resistance, and finally overcoming drug resistance. In response to microbial infections, these kinds of 3D cultured cells respond physiologically and therefore help in better drug screening.
- Because of the specific 3D growth characteristics of the 3D cultured cells, various experimental drugs/toxins and other agents are required in a higher concentration to kill these cells. On the contrary, the normal, mortal adherent cells grow as a monolayer in 2D cell culture containers and, therefore, are much easier to kill by a low dose of experimental drugs/other agents. 3D cell culture is of immense interest in current biopharmaceutical industries and cancer biology studies.
- The spheroids formed by the 3D culture consist of several layers of cells. The nutrition, oxygen, and drug transport in the innermost layer of cells of the spheroids are less. As compared, in general, the 2D cultured cells are a single layer of cells with easy access to nutrition, oxygen, drugs, etc. This may be some of the reasons for **hypoxia and chemotherapeutic drug resistance** in the inner layers of 3D cultured cells.
- In vivo studies using animal models are costly and complex with problems of unpredictable characteristics, and ethical approval must be required. Three-dimensional model systems using human cells are closer to the mammalian physiological system and are, therefore, authentic models of an obvious choice (Pampaloni et al. 2007; Chitcholtan et al. 2012).

2.1 Limitations of Three-Dimensional Cell Culture

Three-dimensional cell culture is not without any limitations. Limitations of the individual 3D cell culture methods are discussed in their specific mentions.

Some general limitations of 3D cell culture are as below:

- There is a wide-scale variation of results apart from poor experimental reproducibility between consecutive batches.
- It is difficult to extract the cells in most of the methods.
- Construction of a 3D culture matrix is difficult and laborious since several components are required to construct a 3D culture matrix. It also necessitates standardization of the concentration and specific ratios of each of the components present in a 3D matrix.
- It is a difficult process, if not completely impossible to scale up or scale down once a formatting architecture is already formed and the cell culture started.
- In 3D culture, taking a clear visible image of the cultured cells depends upon several factors that include transparency of the materials used, size of the scaffold, the depth of the microscope, and others. Therefore, chances of getting clear images of the culture may be limited.

- In 3D culture, a lot of standardization is necessary to check the performance, sensitivity and compatibility, and the sensitivity or resistance of experimental molecules.
- A lot of standardization is necessary with higher reproducibility, consistence, and also improved stability in long-term experiments for being deemed suitable as a routine tool.

3 Materials and Instruments Used for Three-Dimensional Cell Culture

Mainly five types of platforms are used for 3D cultures (Cukierman et al. 2001; Ma 2004; Lee et al. 2008). They are as follows:

1. Scaffolds
2. Scaffold-free platforms for spheroid growth
3. Gels
4. Bioreactors
5. Microchips

Here is a brief description of each of these platforms.

3.1 Scaffolds

In 3D cell culture and tissue engineering, a scaffold denotes a highly porous polymeric biomaterial structure (**matrix**) that acts as a 3D platform to facilitate cell growth and tissue regeneration. Thus, scaffolds are also called **3D matrices** (Chan and Leong 2008).

3.1.1 Properties of Scaffolds

The most important properties of scaffolds are as follows:

Biocompatibility
Biodegradability
Mechanical properties
Scaffold architecture

Here is a brief discussion about the above properties.

Biocompatibility

- The very first criterion of any scaffold for tissue engineering is that it must be biocompatible. A selected scaffold must enable cell adherence, normal functioning, surface migration, and eventually through the scaffold proliferation before laying down a new matrix.

- The implanted scaffold must be immunologically compatible and tolerated by the host's immunological cells. This would prevent inflammatory response by the host's immunological cells, followed by prevention of rejection reaction.

Biodegradability

- The natural essence of a scaffold mandates its proportionate biodegradability to allow the production of a native extracellular matrix by the cells. Thus, scaffold transplantation is not a permanent solution but allows the body's cellular system and ECM to produce their cells and matrix.
- Once biodegraded, the scaffold-degraded materials must be nontoxic to the human body and therefore exit the human body without any type of interference to the physiological system of the human body.
- There should be a clear correlation between the degradation of scaffolds and the formation of new tissues. This indicates that the degradation of scaffold and synthesis of new tissues must be in tandem.
- Now that tissue-engineering strategies are becoming familiar in clinical practice, the field of immunology is swiftly acquiring prominence in the research domain.

Mechanical Properties

The mechanical properties of the scaffolds should be designed in such a way that they would be matched with the anatomy and other mechanical properties of the implanted site. Maintaining the mechanical properties of engineered bone and cartilage is a difficult task. It must be noted that many materials have been produced with promising mechanical traits that retained high porosity. However, due to insufficient vascularization, these engineered scaffolds failed to respond as and when implanted. Several lines of evidence indicate that a balance between the mechanical properties and porous architecture is necessary to allow cell infiltration and vascularization.

Scaffold Architecture

- The architecture of scaffolds is of critical importance for 3D cell culture and tissue engineering. The scaffold architecture should have resembled the in vivo physiological architecture of the tissues and organs. For the supply of nutrients and oxygens, there should be a sufficient amount of porosity and interconnectivity across the scaffolds. This will help transport and diffuse various molecules across the scaffold which supports easy growth of the cells without any hindrance. The porous structure also easily eliminates the metabolic wastes of the growing cells. Gradually the scaffold should degrade and the newly growing cells produce a new matrix that eventually replaces the degrading scaffolds. Scaffold arch structure should have enough capacity to eliminate the degrading scaffolds easily.

  The interaction between the scaffolds and the cells is mediated by a specific chemical group present on the surface. For example, scaffolds synthesize from the natural extracellular matrix material (ECM)s such as collagen contains the amino acid sequence **RGD (Arg-Gly-Asp).** This amino acid biochemically interacts with the scaffolds**.** Instead of natural ECM materials wherever synthetic materials

are used to synthesize the scaffolds, this type of specialized sequence and ligands must be introduced**.**

- The density of the abovementioned ligands is influenced by the specific surface area, i.e., the available surface within a pore to which cells can adhere. For proper function, mean pore size of the scaffolds is important. Thus, the pore diameter must not be too large to prevent the availability of a sufficiently high specific surface, proportionate to minimal ligand density-facilitated efficient binding of a threshold cell population to the scaffold.
- Summarily, a critical pore size range exists for scaffolds, and choosing a particular pore size depends upon the cells, and tissue used for 3D culture.

Scaffold architecture can be briefly expected to have the following characteristics:

- Highly porous.
- Optimum pore size for cell penetration and tissue impregnation.
- The nutrients and gaseous exchange should be permeable through the porous scaffolds.
- Provide an appropriate stress environment.
- Surface conducive toward cell attachment.
- Promote extracellular matrix production and deposition.
- Carry and transmit biomolecular signals.

3.1.2 Materials Used in Scaffold Preparation

- A range of materials (natural and synthetic, biodegradable, and permanent) have been studied for their feasibility to make scaffolds. Examples of such materials include collagen and some polyester (Nikolova and Chavali 2019).
- A commonly used synthetic material is poly-lactic acid. This polyester degrades within the human body to form lactic acid, which can be utilized by natural cellular metabolic pathways (**CORY CYCLE**).

Scaffolds can also be constructed from natural materials, in particular from manifold derivatives of the extracellular matrix.

- Protein materials such as collagen, fibrin, and polysaccharides like **chitosan** or **glycosaminoglycan (GAGs)** have been demonstrated significant and optimal in terms of cell compatibility, but some concerns with potential immunogenicity remain to be resolved.
- Through the bioengineering process, new types of biomaterials have been developed. These biomaterials are biocompatible, nonimmunogenic, transparent, and injectable with a low absorption rate. At present, several nanoscale fibers have been developed that work in low concentrations.

Here is an important discussion regarding the scaffold materials:

- The best scaffold for 3D cultured cells ideally comprises the target tissue **extracellular matrix (ECM)** in its native state. In general, the function of ECM is important for deciding the tissue phenotype, mechanical stability, cell motility, proliferation, differentiation and morphogenesis, intra-/intercellular signaling, and repair ability. Thus, the ECM plays a key role in tissue homeostasis, and sustenance of normal, pathological, and malignant states.
- The common matrix material used in the 3D cell culture system is **collagen.** There are several reasons for the preferential use of collagens as a 3D cell culture matrix material. Collagens are the most commonly occurring matrix materials in the human system. Careful analysis shows that changing collagen concentration or inducing collagen cross-linking can vary the pore size, ligand density, and stiffness of the scaffolds.
- Additionally, it is easier to process the collagen-made scaffolds with flexible manipulation and low cost.
- Besides collagen, other most commonly used materials for making scaffolds include **agarose, fibronectin, gelatin, laminin, and vitronectin.**
- A variety of **synthetic materials** including **metals, ceramics, natural and synthetic grade polymers,** and **composites** are also used to prepare scaffolds.
- At present, the types of matrices and scaffolds are more than 100 in number. The selection of the specific types of scaffolds to be used depends upon the cell type and the nature of the study.
- The major techniques used to produce matrices and scaffolds are **3D printing, electrospinning, or particulate leaching.**
- The most important properties of scaffolds for clinical applications include **biocompatibility, wettability, mechanical properties,** and **surface chemistry.**
- These characteristics of a scaffold depend on its **porosity, pore sizes, permeability,** and **mechanical response-ability. Optimum** porosity aids in adequate **mass transport of nutrients, oxygen,** and **wastes** allowing a larger culture growth than the scaffold-free platform.
- The fabrication methods may introduce a random or ordered structure in the native scaffold texture and therefore must also be considered an important property of scaffolds.

The two main application domains of scaffolds are as follows:

1. For clinical and regenerative medicine applications use of functional implants
2. for manifold laboratory applications in vitro three-dimensional scaffolds

NB: It can be difficult to extract all cells for analysis with increased scaffold size and tortuous order.

- Imaging may also become difficult depending on the **scaffold size, material transparency,** and microscope **depth**.

- In a 2008 review article, *Lee and colleagues* discussed the macro-, micro-, and nano-scale attributes of 3D scaffolds. Readers are suggested to consult this literature source for the morphology-dependent distinctive functional abilities of scaffolds.

  In the delivery of small molecules including drugs to specific tissues, the functional groups of scaffolds may be useful.

Here are some examples of absorbable and nonabsorbable scaffolds:

Absorbable and Nonabsorbable Scaffold Receptivity

By nature, the scaffold may be absorbable or nonabsorbable.

1. Materials Used for Making Absorbable Scaffolds
   Synthetic polymers
   Natural polymers
   Natural minerals
2. Materials Used for Making NonAbsorbable Scaffolds
   Synthetic polymers
   Synthetic ceramics

3.1.3 Examples of Biomaterials Used as Scaffolds

Collagen
Gelatin
Hyaluronan
Poly-glycolic acid (**PGA)**
Poly-lactic acid (**PLA**)
Poly-DL-lactic-co-Glycolic acid (**PLGA**)

Here is a brief description of the biomaterials used for the preparation of scaffolds.

Collagen

This complex protein is made up of three polypeptide chains and contains a **triple helix structure.** The major amino acids present in the collagen are **glycine, proline, hydroxyproline, and arginine**. Around one-third of all the human body proteins are occupied by collagen. Biochemical analysis showed around 60% of gingiva-connective tissue and 90% of total bone proteins.

Gelatin

- It is a mixture of peptide and protein which is made from **partial hydrolysis of collagen**.
- Since collagen is highly present in the animal-connective tissues, skin, and bones, these are the primary sources of gelatin.
- The main animals used for the isolation of collagen are cattle, chickens, pigs, and fish.

- The chemical composition of gelatin is closely similar to collagen. Like collagen, gelatin has **glycine, proline**, **and hydroxyproline** amino acids in its polypeptide chain.
- Glycine is responsible for the close packing of polypeptide chains while proline restricts the conformation.
- Just like collagens, gelatin is also used in the cell culture laboratory as an adhesive agent for adherent cells.

Hyaluronan

This linear polysaccharide is also known as hyaluronic acid. **It is an anionic, no-sulfated molecule and consists of repeating units of glucuronic acid and N-acetylglucosamine linked via alternating β-1,4 and β-1,3 glycosidic bonds**. Hyaluronan is widely distributed throughout connective, epithelial, and neural tissues.

Poly-Glycolic Acid

Poly-glycolide or **poly-(glycolic acid)** (**PGA**) is a simple, linear aliphatic polyester (polymer) with biodegradable capacity. For the preparation of this polymeric compound, either ring-opening polymerization or poly-condensation of **glycolic acid** has been used (structure of glycolic acid: Fig. 1).

Poly-Lactic Acid

Poly-lactic acid is a thermoplastic aliphatic polyester with biodegradable capacity. It is derived from renewable biomass, from fermented plant starch such as sugar

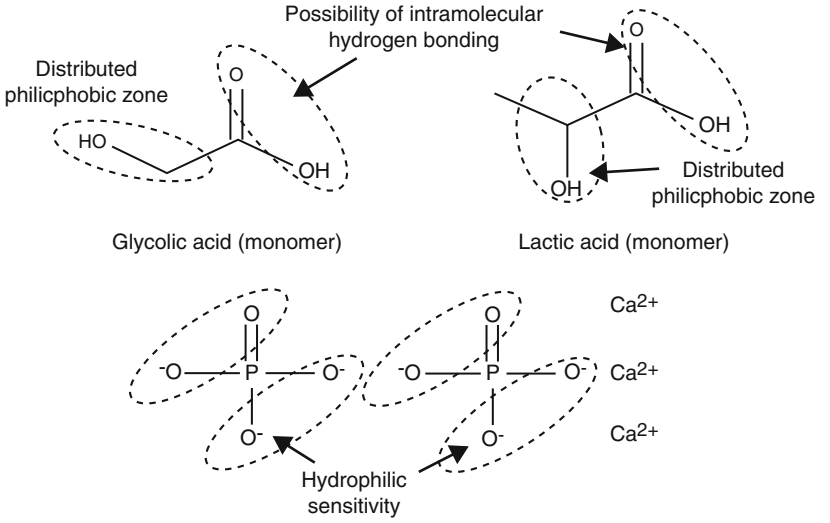

Fig. 1 Monomeric units of poly-glycolic and lactic acid assemblies, manifold OH groups in both monomers allow a hydrogen bonding-manifested water solubility along with distributed philicphobic character to avoid excess aggregation

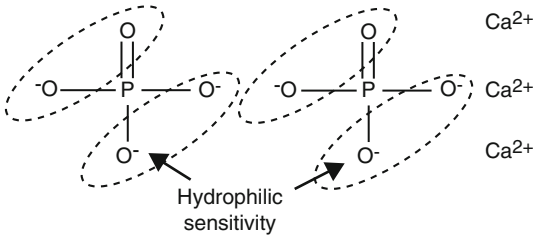

Fig. 2 Chemical structure of tricalcium phosphate, signifying its hydrophilic sensitivity for an in vivo suitability

beet pulp, cassava, corn, cassava, or sugarcane. All these compounds are called **renewable biomass**. In 2010, among the global bioplastics consumption, **poly-lactic acid** had the second-highest volumetric. Figure 2 depicts the monomeric unit of poly-lactic acid assembly for distributed hydrophilic and hydrophobic interaction sensitivity.

Poly-DL-Lactic-Co-Glycolic Acid

This is a biodegradable polymer with high physical strength and biocompatible capacity. It is widely used as a delivery vehicle for DNA, RNA, proteins, various drugs, experimental toxins, and other molecules. Thus, it is established as one of the best-used biodegradable polymers.

Synthetic Ceramics

Used as matrix materials for facilitating in vivo regeneration. The most widely used forms are **tricalcium phosphate** and **hydroxyapatite.** Figure 2 depicts the chemical structure of tricalcium phosphate, where the six negatively charged and two neutral oxygens, along with three Ca^{+2}, confer a hydrophilic sensitivity and hence an in vivo suitability of use as a support matrix agent.

Tricalcium Phosphate

- Porous form of calcium phosphate
- β-TCP
- Susceptible from physicochemical dissolution after implantation

Synthetic Hydroxyapatite

- Development: alternate form of bioceramic
- Rationale: mineral naturally occurring in bone in hydroxyapatite

Synthetic Polymers

- These compounds degrade by the process of **hydrolysis.**
- The fast degrading compound is **polyglycolic acid.**
- **A mixture of polyglycolic acid and L-lactic acid with a molar ratio of 90:10 produces the copolymer polyglycan 910.**
- The poly-lactic acid is stable under in vitro conditions. Crosslinking of additional D-lactic acid in this molecule resulted in its rapid degradation.

3.1.4 Methods Used for Scaffold Synthesis

The following paragraphs describe the various methods for the synthesis of tissue-engineered scaffolds (Ma 2004; Lu et al. 2013).

A. Nanofiber self-assembly
B. Textile technologies
C. Solvent casting and particulate leaching
D. Gas foaming
E. Emulsification/freeze drying
F. Thermally induced phase separation
G. Electrospinning
H. CAD/CAM technologies
I. Laser-assisted bioprinting

Nanofiber Self-Assembly

- Characteristically, **"nanofibers are lightweight with small diameter, controllable pore structures having high surface-to-volume ratio."** At present, these molecules are used not only in tissue engineering but also in energy storage, protective clothing, filtration, and sensors.
- The techniques used for nanofiber synthesis are **electrospinning, self-assembly,** and **phase separation.**
- For making nanofibers, electrospinning is the most widely studied technique. It provides the most promising results for tissue-engineering applications. The methodology comprises the deposition of a monomer-stabilizer mix on a non-reactive aluminum foil under the influence of an externally applied potential difference. The entire operation is conducted in a vacuum with a provision to periodically remove the spent webbings of the polymer mix. The flow rate of polymer mix and temperature of movement can be optimized. In the beginning, the stoichiometric optimum extent of viscosity enhancer is also added to ensure null aggregation in the polymer mix.
- For the synthesis of **peptide nanofibers and peptide amphiphiles**, the **self-assembly** methodology is currently in use. The driving forces used to synthesize nanofibers are hydrogen bonding, van der Waal forces, hydrophobic interaction, and ionic interactions. Of note, these are the noncovalent forces that are required to fold a protein. Additionally, the pH and the ionic strength of the solution affect peptide nanofiber synthesis (Wei and Ma 2006; Yoshii et al. 2011).

Textile Technologies

In this technique, PLA, PGA, and other semicrystalline polymers are processed into polymeric fibers with biodegradable capacity.

These biodegradable polymers such as PGA either alone or in combination with other biodegradable polymers are widely used in tissue engineering for the synthesis of a heart valve, blood vessels, intestine, cartilage tendon, ureter, and other tissues.

NB: Several limitations of this scaffold impacted its successful use. The major limitations are difficulty in controlling pore shape, fast degradation rate, low mechanical strength, and limited fiber diameter variations (Akbari et al. 2016).

Solvent Casting and Particulate Leaching

- This is one of the important techniques used in the fabrication of scaffolds for tissue engineering.
- Briefly, in this technique salt is first ground into small particles.
- In the next step, the desired size particles are transferred into a mold.
- The salt-filled mold is cast with a polymer solution.
- Evaporate the solvent.
- Leaching away the salt crystal using water to form the scaffold pores.
- In this process, the pore size control by varying the size of salt crystals and that of porosity via salt/polymer ratio.
- The major drawback of this technique is pore shape, and inter-pore openings are not controlled (Intranuovo et al. 2014).

Gas Foaming

- In this scaffold fabrication technique, the "solvent-free formation of porous materials through the generation of gas bubbles within a polymer" is permitted.
- The polymer is molded by high-temperature compression molding of the polymer into a solid disc.
- Then the mold is pressurized with a CO_2/another gas chamber for several days until the polymer is saturated.
- During this period, the gas infiltrates the polymer, creating pores for tissue in growth.
- Finally, if required sensitive molecules can be incorporated into matrices without drastically reducing their bioactivity.
- An advantage of gas foaming is the absence of caustic solvents so that their residual presence has no critical role in the final scaffold makeup, making it feasible to incorporate sensitive bioactive molecules (Salerno et al. 2009).

Emulsification/Freeze Drying

It is one of the important techniques of scaffold fabrication. This is a "**sublimation process** in which the frozen water in the polymer nanocomposite is directly converted from solid to gaseous form without apparent liquefaction."

Thermally Induced Phase Separation

- For obtaining a well-interconnected porous structure of scaffold formation, the Thermally Induced Phase Separation (**TIPS**) is a widely adopted procedure.
- Preparation of **demixing solution** of a homogeneous polymer solution is an important characteristic of this technique.
- For demixing, the temperature is changed and the homogeneous solution is separated into a polymer-rich and a polymer-less phase.

- Either **a liquid-liquid** (usually for ternary polymer/solvent/nonsolvent mixtures) or **solid-liquid** (usually for binary polymer-solvent mixtures) demixing occurs.
- Using this technique, it is possible to obtain a well-interconnected polymer network with an easy-to-tune, fast, and adaptable process.
- This technique produces a wide range of morphologies such as fibrous structure, open or close pores, membrane/like architecture, etc.
- Examples of polymers used in this technique are poly-caprolactone, polyurethane, poly-lactic acid, etc. (Gong et al. 2006).

Electrospinning

- Electrospinning was first introduced in the early 1930s to fabricate industrial or household nonwoven fabric products. The technique has been rejuvenated over the past decade to process biodegradable and/or biocompatible polymers (macromolecules) into fibrous textures with an average fiber diameter on the micrometer or nanometer scale for tissue-engineering scaffolds.
- This kind of fiber is formed by a polymer solution forced through a capillary, forming a drop at the tip.
- Between the tips and a grounded collection target, a high voltage is applied. A polymer solution jet is initiated and accelerated toward the collection target as and when the electric field strength overcomes the surface tension of the droplet. As the jet travels through the air, the solvent evaporates leading to the deposition/imprinting on the target (Fig. 3).

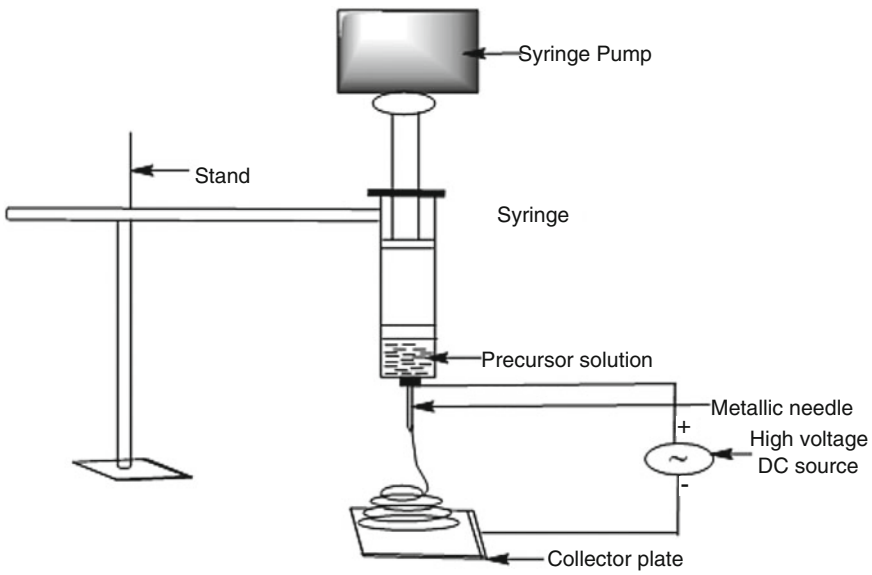

Fig. 3 Experimental setup for "Electrospinning" instrumentation, the speed of precursor solution, and its deposition rate on the collector plate can be controlled by varying the applied electrostatic DC voltage

- An electrically grounded rotating drum is used as the collection target which generates preferential orientation or/and tubular structure formation. The natural macromolecules such as collagen and fibrinogen or certain synthetic polymers such as PGA, PLGA, PCL, and synthetic polypeptides have been processed into fibrous nonwoven scaffolds.
- However, there are challenges like maintenance of desired vacuum pressure, polymer solution-optimized formation to fabricate complex three-dimensional scaffold shapes, and internal pore networks.
- In addition, the average fiber diameter is usually on the larger side of the extracellular matrix fibers, sometimes falling in the micrometer range (Saraf et al. 2010; Sun et al. 2007).

Computer-Aided Design with Computer-Assisted Manufacturing Technologies

Further, the advantages of Computer-Aided Design (**CAD)** with Computer-Assisted Manufacturing (**CAM**) are of particular interest to tissue engineers to reproduce complex scaffold architectures without any need for molds. While the engineering potential of various scaffold architectures is considerable, the ability to design and optimize structures is still very much ad hoc science, with immeasurable local structure and mechanical/transport properties during tissue growth in vitro or in vivo. Hence, CAD allows the systematic design of different scaffolds, making it feasible to monitor the characteristic effect of modulating stoichiometry.

The application of computer-aided technologies in tissue engineering is summed up as "Computer-Aided Tissue Engineering (**CATE**)."

The application of CATE to the design and fabrication of tissue scaffolds can facilitate the authentication of many novel ideas for incorporating biomimetic and biological features into the scaffold design.

The various CATE approaches are as follows:

- **Computer-Aided Tissue Engineering includes the following:** Bioblueprint modeling for 3D cell and organ printing, scaffold modeling and design, and solid freeform fabrication of tissue scaffolds.
- **Computer-Aided-Tissue-Informatics includes the following:** "computer-aided tissue classification and application for tissue identification and characterization at different tissue hierarchical levels."
- **Computer-Aided-Tissue-Modeling includes the following:** "3D anatomic visualization, 3D reconstruction, and CAD-based tissue modeling" (Alghazzawi 2016).

Laser-Assisted Bioprinting

- "3D printing or additive manufacturing (**AM**) is capable of generating scaffolds, in line with the patient-specific requirements."
- The additive manufacturing used by 3D bioprinting produces 3D structures of active biomolecules, biomaterials as well as living cells.

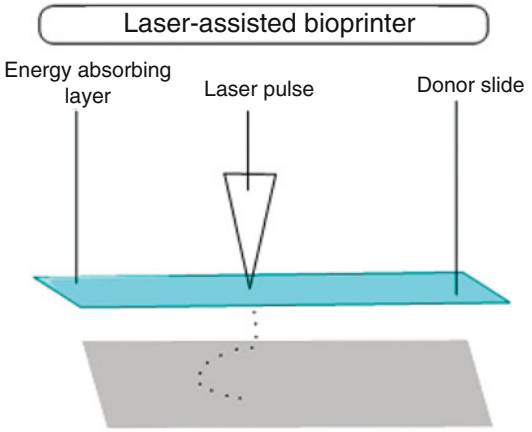

Fig. 4 Typical setup for laser-assisted bioprinting instrumentation

- In large scaffolds with a large number of cell-seeding capacity, the homogenous cell distribution across the scaffolds may be an issue. These kinds of difficulties can be overcome by bioprinting.
- At present, bioprinting technology is based on **laser AM technology** or **inkjet extrusion.**
- **The basis of bioprinting is the use of hugely in the form of bioink.** Of note, 3D material with molecular network hydrogels are equipped with a high water content which allows for cell entrapment and encapsulation without inflicting damage to the cells. Therefore, hydrogels are most commonly used as the base material for bioinks (Fig. 4).
- During the development of bioprint, the main parameters that need to be considered are the material that one has chosen, the concentration of the chosen materials, and its chemical properties.
- The main considerations for bioinks have been explored in a review by *Prendergast and colleagues*. Most significant present-day bioink usage involves a thorough understanding of bioprinting techniques alongside the latest paradigms in the development of bioprinted tissue scaffolds (Guillotin et al. 2010).

3.1.5 Functional Characteristics of Three-Dimensional Scaffolds

A 3D cell culture scaffold may have the following functional characteristics:

Architecture
Cyto and tissue compatibility
Bioactivity
Mechanical response

Here is a brief detail of the abovementioned scaffold-functional characteristics:

Architecture

- A scaffold should provide enough space for new cell/tissue formation, remodeling, and vascularization.
- A scaffold must be porous enough for efficient nutrient and metabolite transport without significantly compromising mechanical stability.
- The biomaterials that are used to make scaffolds must degrade at a rate matching that of the new matrix formation by the dividing cells or developing tissues.

  NB: However, the geometric match and biodegradability analysis are less necessary when scaffolds are used for* in vitro *laboratory applications.
- Undesired monitoring of the biodegradability of the scaffolds matching with the new matrix formation is necessary since the degrading product/byproduct may change the pH and chemistry of the cell culture system and therefore may affect the growth of the cells.
- Additionally, it may be difficult for the growing cells to retain their 3D configuration when the scaffolds are consistently degrading and reorganizing due to the consistent synthesis of biomaterials by the growing cells.
- Therefore, scaffolds should represent a stable structure and function similar to the natural in vivo environment.

Cyto and Tissue Compatibility

The biomaterials used to make scaffolds must be compatible with the cellular components of the cells/tissues to be cultured.

Bioactivity

- The biomaterials of the scaffold should be chosen in such a way (e.g., adhesion molecules) which could augment the cross-talk between the scaffold and supported cells/tissues.
- Some scaffold biomaterials may be the source of growth factors and other nutrients, a controlled release of which may facilitate a suitable growth pace.

Mechanical Property

- The mechanical properties of the scaffold biomaterials should match the need for cultured cells/tissues.
- Several publications reveal that the 3D cultured cells (e.g., epithelial cells) sense the stiffness of scaffold biomaterials and accordingly change their morphology and adhesive characteristics.

3.1.6 Types of Scaffolding Approaches Presently Used

At present, four major scaffolding approaches are in use. They are as follows:

- Implanting cell-seeded premade porous scaffolds
- Implanting cell-seeded decellularized allograft or xenograft ECM
- Implanting laminated cell sheets with secreted ECM
- Injecting cell-encapsulated self-assembled hydrogels

NB: The details of these scaffolding approaches can be traced to the 2008 comprehensive contribution of Chan BP and colleagues.

3.1.7 Applications of Scaffolds

The scaffolds have the following functions:

- The scaffolds allow the cultured cells to attach, and retain the cells and biochemical factors.
- It allows the attached cells to grow and migrate**.**
- The vital nutrients and the products expressed by the cultured cells easily diffuse through the scaffolds.
- Scaffolds can exert certain mechanical and biological influences to modify the cell phase behaviors.
- In general, scaffolds are biodegradable and preferably absorbed by the surrounding tissues without the necessity of surgical removal (Ma 2004).

3.2 Scaffold-Free Platforms for Spheroid Growth

- Cellular spheroids are formed by spontaneous aggregates of different cell types.
- Therefore, spheroids are self-assembled spherical clusters of cell colonies.
- Spheroids are either self-assembled or are forced to grow as cell clusters (e.g., pellet culture) starting from single-cell suspensions.
- Scaffold-free platforms have no support structure or porosity.
- Cells that grow on scaffold-free platforms as spheroids generate and organize their own ECM. Therefore, spheroids are closely evolved into the in vivo tissue systems.
- Large spheroids are about **500–600 μm in diameter,** and it is not possible to grow spheroids larger than this diameter.
- These large spheroids have a characteristic growth pattern resembling the **tumor** and **cancer cell heterogeneity growth pattern**, i.e., **an external proliferating zone, an internal quiescent zone characterized by limited oxygen availability, nutrient, metabolites distribution zone, and a necrotic core.**
- In a spheroid, **coculture** of several cells is possible and thus prediction of the multiple toxins and other drug effects is more realistic compared to 2D cell culture.
- Analysis of the spheroid structure shows that it resembles the structure of **embryoid bodies, avascular tumors, and solid tissues.** Therefore, this type of structure shows a significant application in cancer and stem cell research.
- The metabolic and proliferative gradient of spheroids is closely similar to **physiological models.**
- The harvested spheroids can be analyzed using **luminescence assays, fluorescence assays,** or **colorimetric assays** using a plate reader.
- In case microscopic images of a spheroid are necessary, it can be easily performed by putting it on a transparent plate, lid, and tray assembly.

- "The platform also offers simplified liquid handling procedures and compatibility with HTS instruments, through liquid handling robots like the Biomek® FX and epMotion automated pipetting systems."
- Both for basic laboratory research and high-throughput screening (**HTS**) applications spheroids are applicable (Baraniak and McDevitt 2012; Klingelhutz et al. 2018).

3.2.1 Techniques to Form Spheroids and Microtissues

Various culture methods related to spheroid culture are discussed below:

Pellet culture method
Spinner culture method
Hanging drop method
Liquid overlay method
Rotating wall vessels method
External force method
Cell sheets method
Microfluidics method
Micromolded nonadhesive hydrogels method

Here is a brief discussion of the above cultural procedures.

Pellet Culture Method

The specialty of this technique is to maximize the cell-to-cell adhesion followed by spheroid formation by using centrifugal force. Of note, the centrifugal force concentrates the cells at the bottom of the culture tube and thereby enhances the chances of accumulation of the cells at the bottom of the culture tube and increases the chances of cell adhesion. Thus, the name pellet culture originates (Fig. 5).

Very briefly, the process is as follows:

- Make a cell suspension.
- Transfer the cells to a tube with a conical bottom.
- Incubate the cell suspension in a shaker for an hour.
- Centrifuge the tube at 50 rpm for 5 min.

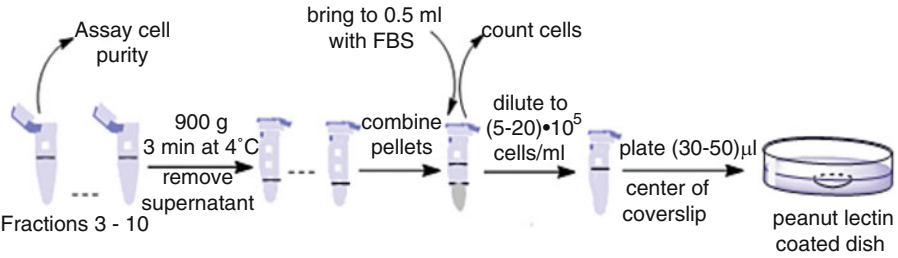

Fig. 5 Sequential stages of pellet culture method

- The major advantage of the procedure is it is an easy procedure.
- However, there are some disadvantages to this technique. The major disadvantage is the formation of **shear force** that is generated due to centrifugation which is deleterious for the mammalian cells.
- Another disadvantage is the formation of low oxygen concentration or **hypoxia** at the center of the spheroids due to formation of large spheroids.
- The method is also not optimized for the large-scale production of the spheroids.
- Some of the widely used cell cultures that use this procedure are **differentiation of mesenchymal stem cells (MSCs), bone formation, and chondrogenesis**.

"***For chondrocytes, differentiation is stimulated by low oxygen, but for other cell types, hypoxia can cause necrosis in the spheroid core (Jahn et al.*** 2010***)***."

Spinner Culture Method

- The specificity of the spinner culture method is to constantly stir the cell suspension and thereby decrease the chances of cell sedimentation which enhances the cell-to-cell contact or collision followed by spheroid formation.
- In this procedure, a well-mixed single-cell suspension is put into a spinner flask and an impeller or magnetic stirring bar is constantly agitated to form the **convective forces** (Fig. 6).
- The major advantage of this procedure is the transport of oxygen and nutrition at the center of the spheroid due to constant agitation.
- This culture procedure is used for various primary culture cells and cell lines, as well as **heterotropic spheroid** formation by the mixture of two different cell lines (Collins et al. 1998).

Drawbacks of the Spinner Culture Method

- Maintaining the constant speed of the agitator is critical for this technique. The too low agitation speed will allow the cells to settle down at the bottom of the tube. On the other hand, the high speed of the impeller will create a shear force that will be harmful to the cells.

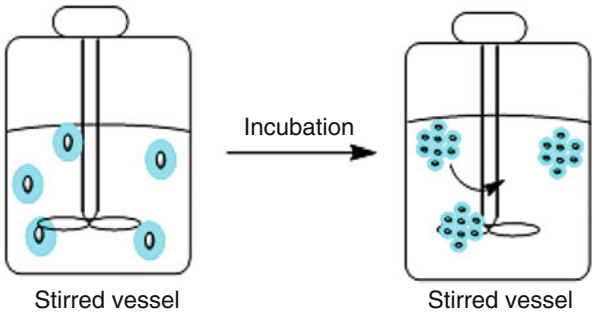

Fig. 6 Schematic description of spinner culture method

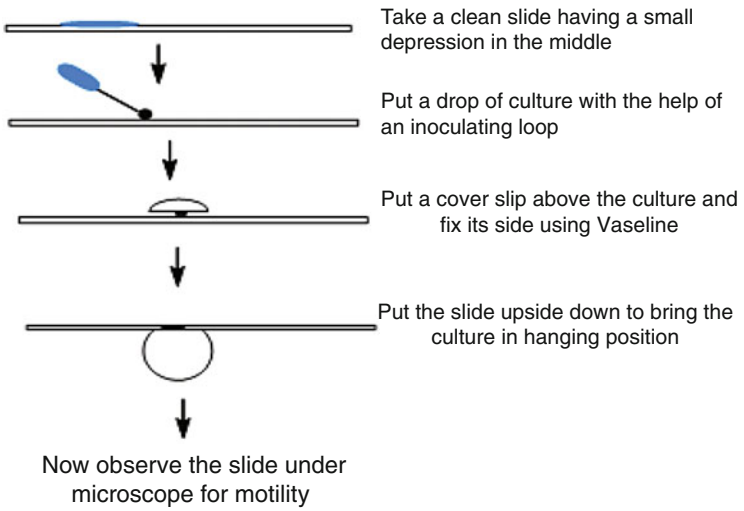

Fig. 7 Sequential steps in hanging drop mammalian cell culture approach

- Due to constant mixing (spinner action), cells cannot be visualized as they **form aggregates**.
- Adherent cells cannot be used in spinner culture since they **undergo apoptosis in suspension**.
- In this procedure, spinning of the culture creates **shear forces** that may damage the cells, particularly those having **low cohesiveness**.

Hanging Drop Method

- In this method, a small drop of plasma or some other fluid is suspended from an inverted watch glass to allow the growth of the cells/tissues. The generation of hanging drop minimizes the surface area to volume ratio and thus slowing down the evaporation of the liquid used for the culture (Fig. 7).
- One of the important advantages of this method is gravity-enforced self-assembly to form the spheroids allowing tissue growth, suspended from an inverted watch glass.

Briefly, the procedure is as follows:

- **20–30** μl of cell suspension is pipetted onto the inside lid of a tissue culture plate.
- In the next step, the lid is inverted; however, due to **surface tension,** the liquid drop remained attached to it.
- Because of gravity, the cells settle and concentrate at the bottom of the drop, allowing the cell-to-cell contact and formation of a single spheroid. In this case, heterotrophic cells can also be used.
- Adjusting the cell density of each drop, one can control the spheroid size and cellular composition.

- Standardization of a large number of primary cultured cells as well as cell lines is completed.
- "Advances into high throughput spheroid production using the hanging drop method have been made, producing up to 384 spheroids in a single array (Foty 2011; Tung et al. 2011)."

Disadvantages of Hanging Drop Method
- The major drawback of this method is the difficulty to track the spheroids during formation.
- To exchange medium or add drugs is nontrivial.

Liquid Overlay Method
- The specificity of this method is to make a flat tissue culture dish with non-adhesive properties by adding a thin layer of **agarose and poly (2-hydroxyethyl methacrylate) (pHEMA).** This layering of **agarose and pHEMA inhibits the adhesion of cells with the culture plate.**
- In the next step following seeding, the cell suspension in this nonadhesive culture, the plate is rocked or shaken leading to the aggregation of cells (Fig. 8).
- The spheroids formed in this procedure are **heterogeneous both in size** and **shape.**
- **This technique is utilized to form coculture spheroids primarily consisting of osteoblasts, fibroblasts, and endothelial cells.**
- As and when the spheroid culture is performed in 96 well, plate monitoring of the individual spheroids is possible.
- A large number of spheroids are formed by this technique both from primary culture and from the various cell lines.
- When carefully monitored, it was observed that there are variations in morphology, thickness, growth rate, radiosensitivity, and PO_2 profiles of the cultured spheroids.
- This type of variation indicates that different tumors may have different variables and variables may also exist within the different regions of single tumors.
- These factors if carefully monitored and standardized may be the sources of significant information development of a tumor and cancer cells and may be helpful in the development of suitable cancer therapy (Costa et al. 2014).

Rotating Wall Vessel Method

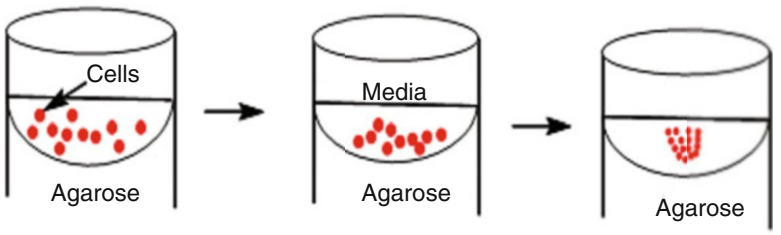

Fig. 8 Schematic description of microwell liquid overlay method

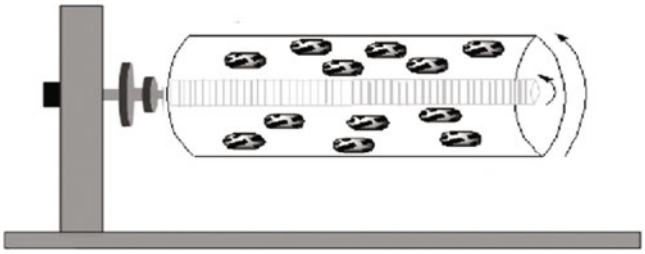

Fig. 9 Schematic depiction of rotating wall vessel mammalian cell culture method

- The specificity of this is the creation of a rotating wall vessel.
- The cell suspension is seeded in a rotating wall vessel, and the vessel **rotates about the x-axis** which **creates microgravity and maintains the cells in a state of free fall**. The cells maintained the suspended state and formed spheroids via aggregation.
- The rotation speed of a rotating wall vessel **at the initial stage is 15 rpm**.
- Later on, the speed increased to **25 rpm to keep the growing spheroids in suspension**.
- One of the important advantages of this method is to produce **low shear force and therefore less damage to the cells during spheroid formation** (Fig. 9).
- Like other methods, both primary cultures, as well as various cell lines, can be cultured by this method.
- The coculture of different cells is also possible by this technique.

"Long-term culture is possible with the culture conditions being controlled by perfusion, useful for controlling differentiation" (Schwarz et al. 1991***; Radtke and Herbst-Kralovetz*** 2012***).***

Limitations of Rotating Wall Vessel Method

- Although this method provides a high yield, there can be variability in spheroid size.
- While some advances in tracking the aggregates have been made, it remains difficult to monitor the real-time spheroid assembly.

External Force Method

- In this method generally, three types of external forces are utilized for attaining a high-density cell aggregation (Fig. 10).

They are as follows:

Electric fields
magnetic force
Ultrasound

- For electric fields, positive dielectrophoresis in a low conductivity iso-osmotic solution is used to concentrate cells.
- For magnetic fields, cells are incubated with magnetically sensitive cationic liposomes (MCLs) containing a magnetite core (Fe_3O_4).

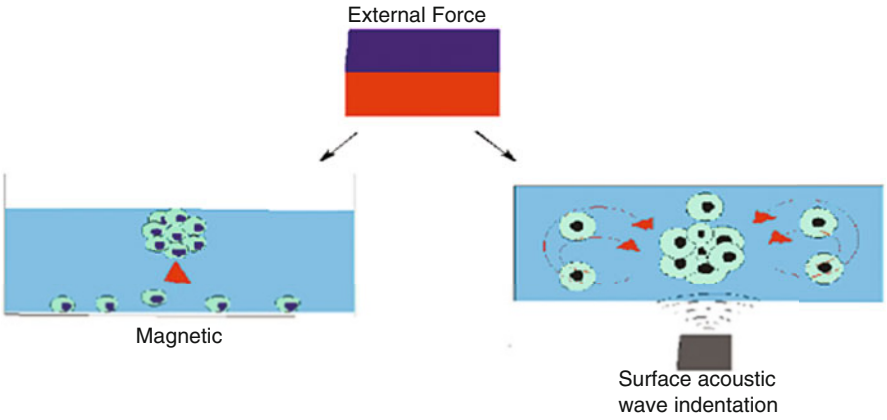

Fig. 10 External force method of mammalian cell culture

- After endocytosis, the cells can be arranged in specific patterns under the influence of magnetic fields.
- A standing wave trap is used to concentrate cells and facilitate their aggregation for an ultrasound.
- It is difficult to control the ultrasound-mediated spheroid size.
- The cell adhesion mediated by the ultrasound is nonspecific.
- The physiological changes of the cells due to ultrasound are not well characterized.

Cell Sheets Method

- In this procedure, **multicellular cell sheets** are produced by **thermo-responsive** polymers, such as **poly (N-isopropyl acrylamide)**. This kind of polymer depicts varied hydrophilicity and hydrophobicity with temperature changes.
- By temperature-controlled hydrophobicity and hydrophilicity changes, one can control the **adhesion and detachment of cells** without the administration of any kinds of proteolytic enzymes.
- For example, to change the polymer from hydrophobic to hydrophilic it is necessary to lower the temperature to 20 °C for 1 h. This eventually facilitates the release of a contiguous cell sheet.
- The terminology indicates that the cultured cells are harvested as intact sheets together with their deposited extracellular matrix (**ECM**).
- "To form spheroids, small cell sheets that have been released can be further incubated on a non-adhesive surface where they compact and form spheroids" (Fig. 11).
- The cells which are most used in this method are **mesenchymal stem cells** for use in cartilage engineering, **hepatocytes, cardiomyocytes, endothelial cells, and epithelial cells.**

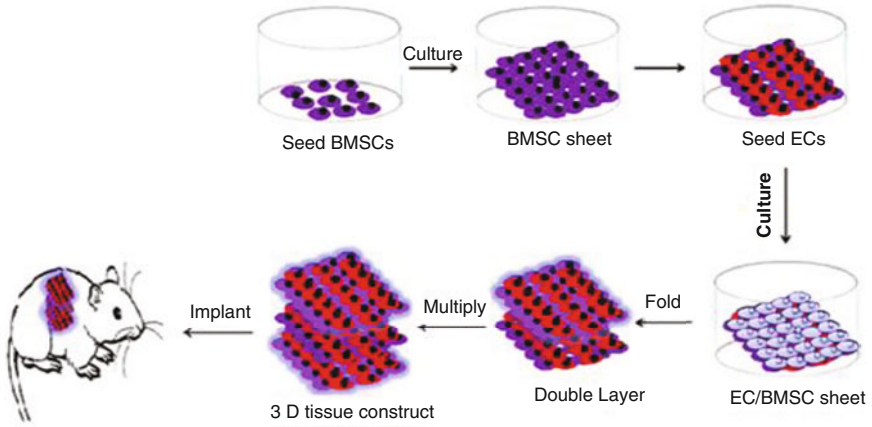

Fig. 11 Cell-sheets method for mammalian cell culture

- "Additionally, co-culture of multiple cell types could be achieved via creating layers of cell sheets from different cell types, and thereafter allowing the multi-layered sheet to assemble into a spheroid" (Sekiya et al. 2013).

Usefulness of Cell Sheet Method

- The most notable advantage of the cell sheet method is the direct transplantation of the cell sheet in the host tissue without a scaffold.
- This method is extensively used to form a 3D organ-like structure.
- The major organ-like structure produced by this method is the heart, liver, and cornea.
- Additionally, this method has been used to generate in vivo tubular circulatory support instruments, as well as in vitro micropumps using myocardial sheets (Alghuwainem et al. 2019).

Microfluidics Method

The name microfluidics originates because of the capacity for manipulation of small fluid volumes (μl, nl, and pl) within artificially fabricated microsystems and cell cultures.

The dimensions of the microfluidic channels are well suited for the cellular physical scale.

Interestingly, the dimension of the eukaryotic cells is between 10 and 100 μm which is well within the range of microfluidic dimensions.

- Experimental observations claimed that the microfluidic cell culture can mimic the cell microenvironment. The microfluidic system may be able to mimic environmental factors that regulate cell structure, function, behavior, and growth.

- Like in vivo cellular systems, the microfluidic system also produces stable gradients. Of note, these gradients play a significant role in understanding durotactic, chemotactic, and hypotactic effects on cells.
- To form cell aggregation and forming spheroids, the cells are allowed to flow through microchannel networks into microchambers where they are partitioned and exposed to microrotational flow.
- This technology is utilized both for primary culture, and culture of cell lines, as well as the culture of multiple cell lines.
- The size-controlled spheroids produced by this technology can be utilized for **high throughput analysis of real-time imaging.** It can be connected with biosensors.
- The additional advantage of this technology is the tight control of the shear stress and the soluble growth factors due to the perfusion of fluids surrounding the spheroids (Whitesides 2006; Mehling and Tay 2014; Bhatia and Ingber 2014).

Usefulness of Microfluidics Method

- The most important advantage of the microfluidic system is reduced sample volume (in some cases, primary cell number is so less that it is not possible to use some other methods to culture).
- In a microscale system, the cells the cell population used may be a few 100 as opposed to macroscale culture where the normal cell number required may be **10^5–10^7 cells.**
- Due to the small volume of the cells, it is easier to study and monitor nondiving or slowly dividing cells.
- One of the major uses of this technique is the compartmentalized microfluidic culture that has been combined with live-cell calcium imaging. In this case, the peripheral terminals of neurons are delivered with the depolarized stimuli and the calcium responses are recorded from the cell body.
- When results were analyzed, it was shown that there is a stark difference in the sensitivity of the peripheral terminals compared to the neuronal cell body toward certain stimuli such as protons.
- Thus, microfluidic technology is very important to study the peripheral terminals in isolation.

Micromolded Noninvasive Hydrogel Method

- For this method, **computer-aided design (CAD)** and **rapid prototyping** are used to form **micromolds**. The micromolds thus formed contain **an array of cylindrical pegs with rounded tops.**

  These micromolds are then cast with nonadhesive hydrogels made up of **agarose** or **polyacrylamide.**

  In the next step, cells are seeded on the micromolded gels. While the cells are settled down at the bottom, they are unable to attach due to the nonadhesive nature of the gels (Fig. 12).

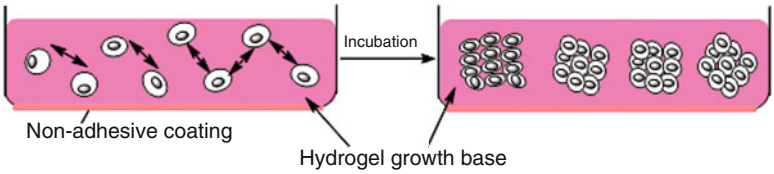

Fig. 12 Growth pattern of micromolded hydrogel supported varying cell shapes

This leads to cell-to-cell interaction culminating in the formation of spheroids. This technology is also utilized to form **microtissues.**

Both primary and secondary cell lines originating from a variety of tissues including skin, brain, ovary, liver, heart, and breast are used in this technique.

Based on the recent estimation, it is claimed that this technology is scaled up to create as many as **822 spheroids** in a single mold.

Careful analysis showed that these spheroids are homogenous in the shape, size, and composition of the cells.

One of the important advantages of this method is some of the important parameters such as shape, size, etc. of the spheroids can be controlled by the **number of cells** and the **ratio of the cell mixture seeded** onto the micromolded hydrogel.

In this method, it is easy to change the cell culture medium or the medium constituents.

Additionally, imaging of the cultured cells is feasible.

- "The micro mold designs could also be used to direct the cellular self-assembly to more complex shapes such as **rods, toroidsor honeycombs** (Geckil et al. 2010; Yanagawa et al. 2016)."

Hanging Drop Method by Gravity-Enforced Assembly

- BioMatrix and other companies customized the 3D hanging drop assembly.
- This simple scaffold-free system can generate consistent sizes and shapes. Thus, testing of spheroids by this technique is controllable and reliable.
- In this technology, one can generate spheroids of different sizes by adjusting the seeding density, from as few as **50 cells** to as many as **15,000 cells**.
- "The plate consists of a main hanging drop culture, a complementary lid and tray, which ensure sterile maintenance with reduced evaporation."
- The fluids and spheroids can be manipulated by the access holes from the topside.
- Around the culture plate periphery, a water reservoir is constructed which allows the evaporation (Fig. 13).

To create the hanging drop standard pipette, tips are inserted into the access holes on the top of the plate and then dispense a small volume of the cell suspension.

In the same way, the drugs and reagents are either removed or added from each hanging drop.

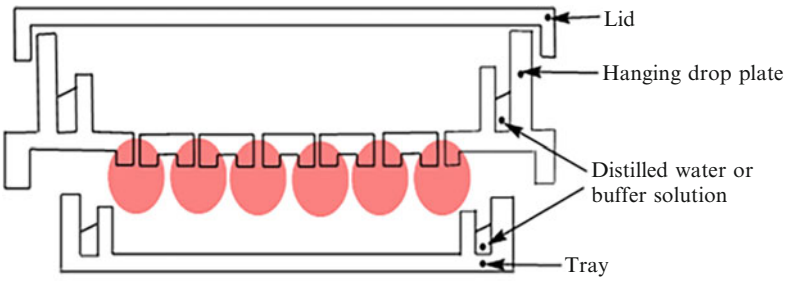

Fig. 13 Hanging drop approach of cell culturing

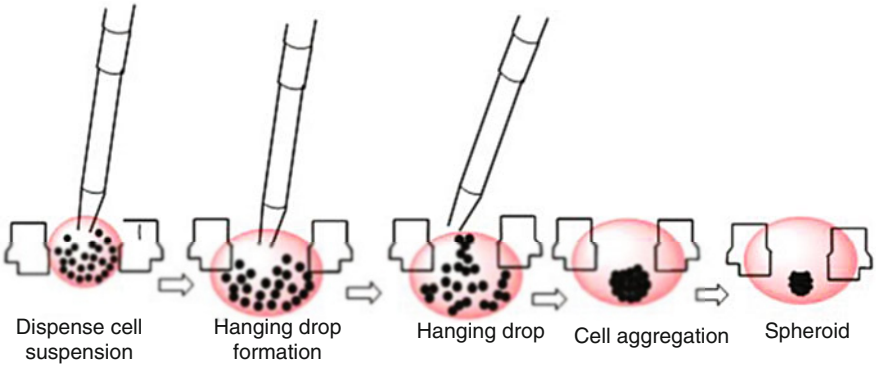

Fig. 14 Perfect 3D hanging drop cell-culturing approach

The hanging drop is stabilized by a plateau structure located at the bottom of the tube. Since a perfect 3D structure hanging drop plates does not attach to the bottom of the plate due to a lack of adhesive substrates, the cell suspension becomes aggregates to form the spheroids (Fig. 14).

Protocol for Hanging Drop

In 2011, Ramsey Foty standardized the following protocol for the hanging drop technique (Foty 2011). The technique is described here with some modifications.

Preparation of a Single Cell Suspension

- Cells with adherent characteristics should be grown up to 90% confluency.
- Twice each of the cells with PBS.
- Remove the PBS.
- In each 100 mm washed cultured Petri plate, add 2 ml, **0.05% trypsin-1 mM EDTA,** and detach the cells.
- After all the cells are detached (~in 3–5 min after trypsin administration), add 2 ml of complete medium to neutralize the trypsin.

- Transfer the cell suspension to a 1.5 ml Falcon tube.
- Add **40 µl DNase (10 mg/ml DNase stock**), mix, and incubate for 5 min at room temperature.
- Centrifuge the tube at 200× g for 5 min.
- Remove the supernatant.
- Wash the pellet with 1 ml complete tissue culture medium.
- Repeat the washing procedure.
- Resuspend cells in a 2 ml complete tissue culture medium.
- Count the cells using a hemocytometer, or automated cell counter, and adjust concentration to **2.5×10^6 cells/ml** (cells as low as **1×10^6 cells/ml** can also be used).
- For counting the cells, a Bio-Rad TC10-automated cell counter could also be used.

Formation of Hanging Drop
- Take a 60 mm Petri plate.
- Remove the lid.
- Add 5 ml PBS at the bottom of the plate.
- Now invert the lid and add **10 µl drops** at the bottom of the lid.
- It is possible to place at least 20 drops per dish.
- Place 10–15 drops, each containing a 20 µl volume on the Petri dish lid.
- Invert the lid over the dish containing 10 ml of sterile water.
- Incubate the dish at 37 °C.
- Within 24–48 h (depending on the cell line used), the cells would have formed the aggregates.
- Using a pipette, gently transfer the aggregates to a fresh plate coated with agar (for preventing attachment) and add a 10 ml medium.
- Incubate the dish at 37 °C, in a 5% environment and with 95% humidity.
- After **24–48 h,** the aggregates that have been unable to adhere to the dish surface would have formed dense spheroids.
- Another option is to seed cells into either a spinner or shake flask at low speed containing 3 ml complete medium, incubated in a shaking water bath at 37 °C and in a 5% CO_2 environment until spheroid formation.
- On becoming confluent, the spheroids generally get formed.

NB: Generally within 24 h, sheet or spheroids may be formed. However, depending upon the cell type it may take a longer time.

3.2.2 Drawbacks in Cells Cultured as Three-Dimensional Spheroids

- Mammalian cell culturing through spheroids is cumbersome.
- It is difficult to control the spheroid size.
- Microscopy and assay of spheroid culture are difficult to analyze.

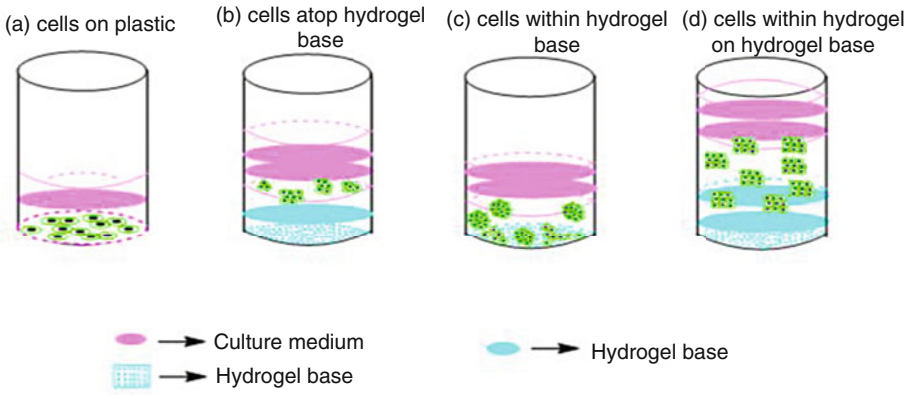

Fig. 15 Typical procedure for hydrogel-based cell culture

3.3 Gels for Three-Dimensional Cell Culture

Natural grade materials such as collagen and alginate are used to make the gels (Jun et al. 2013).

Other materials that are present in the gel are various other ECM materials including laminin, fibronectins, growth factors, enzymes, etc.

Extensive research in this area led to the development of synthetic gels.

For example, **polyethylene glycol (**PEG**)-based hydrogels, from** QGel™**,** are modified to enable desired characteristics.

- **Modification of the gel material can be possible by various methods that include** the incorporation of different proteins or molecules within the matrix, hybridization of natural and synthetic materials, and hybridization of biomaterials with functional nanomaterial (Fig. 15).

3.3.1 Usefulness of Gels as Three-Dimensional Matrixes for Mammalian Cell Culture

- Both natural and synthetic 3D gels are very popular, particularly in cancer research because of their soft tissue-like material properties and the ease of being convertible into 3D cultures.
- Another important aspect of using 3D gels is that several other methods such as spheroids culture, scaffolds, and microchips can be combined with gels and the combination may give better results.

3.3.2 Limitations of Using Three-Dimensional Gels

- Several factors may affect the gelling mechanism and therefore affect their proper usage. For example, matrigel must be kept on ice to keep its viscosity low enough for manipulating and mixing with cells.

- The pH-based gelling mechanism is also common, exposing sensitive cells to adverse conditions.
- Gel constituents may contain contaminated animal viruses.
- Gel constituents may contain nonquantified substances.
- There are batch-to-batch variations in the composition as well as results, leading to difficulty in concluding.

3.3.3 Matrigel

- Matrigel is a product marketed by Corning Life Sciences and BD Biosciences.
- Experimentally, it was shown that matrigel resembles the complex extracellular environment of several tissues. Thus, it is frequently used by cell biologists as a substrate that allows membrane-attached cell growth (Fig. 16).
- For the preparation of matrigel, the reconstitution of the **Engelbreth-Holm-Swarm (EHS) mouse sarcoma** basement membrane extract was used.
- Analysis of the various constituents of the matrigel shows it contains ECM proteins such as collagen IV, laminin, various proteases, nidogen or entactin, and growth factors. Matrigel is one of the most popularly used 3D matrices, utilized for various types of cancer cell research (Kleinman and Martin 2005).

3.3.4 Hydrogels

- Some of the hydrogel characteristics are similar to native tissue such as tissue-like flexibility while possessing viscoelastic properties, interstitial flow, and diffusive transport characteristics similar to native tissues.
- The natural polymeric materials used to form hydrogels are **chitosan, hyaluronic acid (HA), gelatin, fibrinogen, alginate, and collagen.**
- In addition, the synthetic materials used to form hydrogels are **PLA, polyethylene glycol (PEG)** and **PEG derivatives, and PVA** (Jongpaiboonkit et al. 2008; Tibbitt and Anseth 2009).

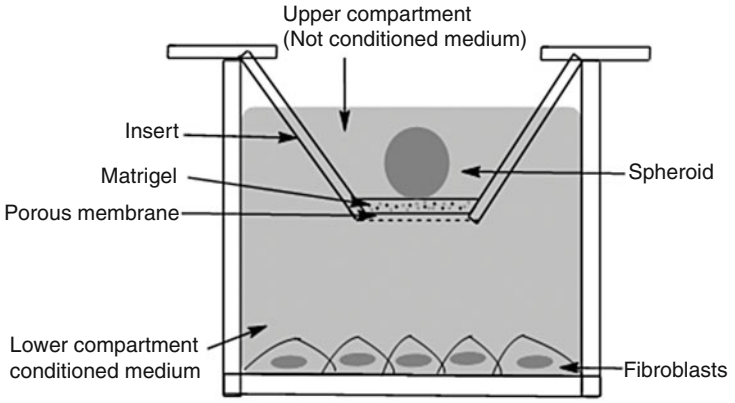

Fig. 16 Representative setup of matrigel (as a nutrient source)-facilitated spheroid culture

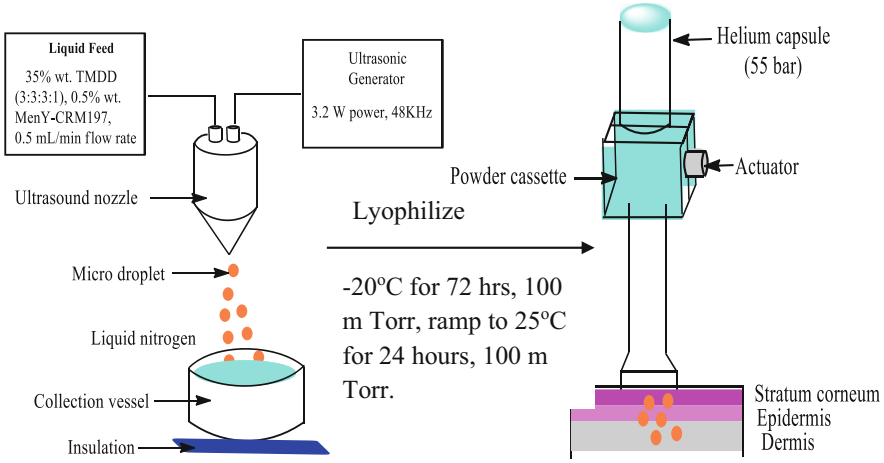

Fig. 17 Typical representation of the freeze-drying process

Methods

Solvent casting and particulate leaching (with slight modifications) are the most common methods used to fabricate the hydrogels.

Gas Foaming

Fabrication of hydrogels using foaming methods is well-characterized and studied. Below are some variations:

Freeze Drying

- First, the polymer is dissolved in a solvent in the desired concentration.
- Next, the materials are frozen.
- Now, the frozen material is lyophilized to remove the solvent.
- Several positive points of the technique are scaffolds that have high porosity and interconnectivity with the pore size controllable through adjustment in the freezing rate and pH (Fig. 17).
- The technique does not require a high temperature.
- However, the method requires a long processing time and often produces smaller pore sizes.

Copolymerization/Crosslinking Methods

Other methods to make hydrogels require **polymer gels, free radical polymerization, in situ polymerization,** and **photo-polymerization**.

Microfluidics

- The use of microfluidics to fabricate hydrogels is an upcoming technology.
- The main advantages of this technique are the product characteristics of uniform pore size, porosity, and complex patterns. Reproducible 3D cell culture systems for hydrogels can be achieved using microfluidics.

3.3.5 Three-Dimensional Growth of MCF-10A in Matrigel

- MCF-10A is the most frequently used **human breast epithelial cell line** (the normal phenotype).
- The cell line is characterized by spontaneously immortalized benign proliferative nontumorigenic breast tissue. The cells do not express estrogen receptors (ERs).
- Karyotype analysis of this cell line showed a depleted chromosomal locus corresponding to housing p16 and p14 ARF genes.
- It is observed that these genes perform critical functions in regulating senescence and MYC gene amplification.
- "Upon being cultured at the top of Matrigel, MCF-10A cells exhibit acinus-like spheroids with a hollow lumen. This structure is enclosed by the basement membrane, constituted of polarized and organized cells."
- 3D cultured MCF-10A cells are used as an ideal model to study the development of mammalian breast, cell-to-cell interaction, and physiological action of epithelial cells as well as specific tissue microenvironment (Lance et al. 2013).

3.3.6 The Standard Protocol for Culturing MCF-10A Cells Using Matrigel Is Chronologically Described as Under

- Take an eight-well chamber slide (BD Falcon Culture Slide).
- Add 45 μl matrigel to each well.
- Spread matrigel evenly inside the well without forming any air bubbles. Do not touch the pipette tips to the edge of the well since this will form a high meniscus on the border.
- Now to allow the thorough gelling of the matrigel, place the slide in a cell culture incubator and keep it approximately for 30–40 min.
- Take freshly cultured (cultured in DMEM/F12 + 20% horse serum + EGF) MCF-10A cells, trypsinize them, and spin down the cells at 900 rpm for 3 min.
- Remove the supernatant.
- Resuspend the cell pellet in a total of 10 ml Assay Medium (same as a 10A growth medium, but with 2% serum and no EGF).
- Count the number of cells.
- Add 400 μl (5000 cells, 2% matrigel, and 5 ng/ml EGF or another stimulant) of cell suspension in the medium in each well on the top of the matrigel.
- "Transfer an appropriate amount of overlay medium (n + 1 wells/cell line × 400 μl) to a fresh tube and add the respective volume of cell suspension."
- Incubate the plates in an incubator and allow them to grow for 10–15 days.
- Every 4 days intervals, replace the old medium with the fresh medium containing 2% matrigel and 5 ng/ml EGF (or another stimulant).
- In about 5–6 days, the cells should form spheres and thereafter form hollow lumen till 7–8 days.

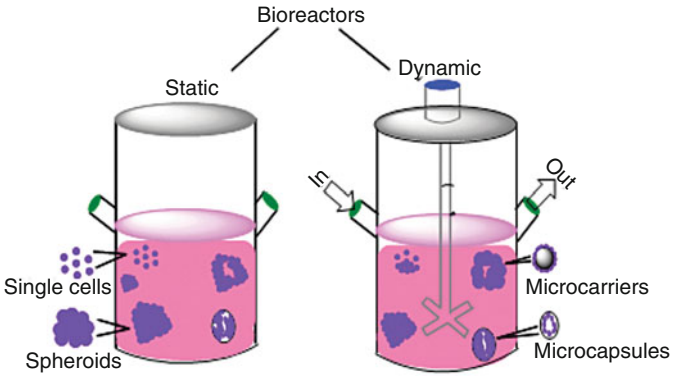

Fig. 18 Static and dynamic modes of a bioreactor

3.4 Bioreactors

A **bioreactor** is typically any device or system that supports a biologically active-controlled environment for the culture of various cell types including those of bacteria, fungus, yeast, and animals.

Bioreactors offer a well-adapted environment for 3D cell culture by the addition of scaffolds and are ideal for high-volume cell production and ex vivo tissue engineering (Fig. 18).

Extensive bioreactors in an application for these requirements comprise media flow, enabling nutrient circulation, elimination of wastes, and accomplishment of an unformed environment. Normally, these reactors are well compatible for cell expansion purposes and designated formation of microbial products, viz. antibodies (Ingram et al. 1997; Edmondson et al. 2014). For more details on bioreactors, readers are suggested to consult the next chapter.

3.5 Microchips

Microchips, frequently as *"organ on a chip"* ***or microsystems, are the next wave of 3D cell culture models. "Organ on a chip" integrates microfluidics technology with cells cultured within microfabricated 3D devices, using microchip industrial techniques*** (Bhatia and Ingber 2014).

- This technology is relatively new and is still in the process of development. However, the ultimate goal of this technology is to join or link all the individual organ microsystems together to develop a human-on-a-chip.
- "Recent advances include development of integrated 'organ on chip' microsystems reproducing key architectural, functional, biochemical and mechanical features such as mechanical strain and shear forces of living organs, including lung, liver, kidney, gut, bone, breast, brain and eye." Readers are suggested to

consult the 2011 publication of Huh and colleagues, to have a better idea in this regard.

- At present, the systems used by microchips are **photo or soft lithography** and **replica-molding techniques** along with **silicone rubber, poly-dimethylsiloxane (PDMS), and microfluidics systems**.
- These systems are optically transparent, highly permeable, and easier to fabricate, as well as less expensive.
- The poor chemical resistance of PDMS toward some solvents can absorb hydrophobic molecules, therefore limiting its suitability in commercial applications.
- The PDMS suffers from poor chemical resistance toward some solvents and can absorb small hydrophobic molecules. These traits limit its commercial application.
- For using microchips, it requires considerable knowledge about the subjects, and these factors limit its widespread use.
- The major application of microfluidic technology is **drug screening, cell-based assay, molecular diagnostics,** and **tissue engineering**.
- The fluids are handled in this technology in the range of **10^{-9}–10^{-18} l.**
- **Digital versions** and **continuous flow** are the two categories of microfluidics.
- "-flow microfluidics can be further categorized as **single** and **multi-phase**. Multiphase microfluidics and digital microfluidics can accurately and efficiently generate micro droplets within milliseconds enabling high throughput for cell-based analysis."
- The application of 3D cultures on a miniaturized scale significantly enables further room in decreasing the nutrient content, reagents, soluble culture additives, and supplemented drugs. Microfluidics enabled cell culturing in three dimensions and confers better control with cost-effective biological fabrication.
- Erstwhile benefits of droplet-driven microfluidics involve cell compartmentalization with a proportionately adequate surface area to volume ratio. This physical prospect is highly suited for numerous molecular and cellular grade utilities. As the cell viability during MCS generation is of high priority, the microfluidic platform makes it feasible with moderated shear stress.
- Apart from the above, the distinguishing attributes of an integrated microfluidic device include the prominent distinctions of programmability and configurability. For instance, cell-based advanced assays could be easily screened as an assay. This trait enables the microfluidics for engineering 3D microscale tissues as well as enhanced complexity systems viz. artificial organs on a chip.

In the following sections, we discuss the state-of-the-art prospects of the following microfluidics systems:

Continuous-flow microfluidics
Single-phase microfluidics
Multiphase microfluidics
Digital microfluidics

3.5.1 Continuous-Flow Microfluidics

- A continuous flow pattern in a microchannel is executed via pressure-/fluidity-regulating pumping systems. On a laboratory scale, fluid flow is characterized by a laminar manner with the pulsating surface phenomenon (like friction) and volume effects (like inertia).
- This mode of microfluidics can access either a single or multiphase segmented flow. The latter approach is often termed droplet-based microfluidics, generating and manipulating monodispersed microscale droplets.
- A single-phase continuous-flow microfluidic device works through hydrogel-coated/-occupied microchannels, which facilitate the functioning equivalent to that of a scaffold to support cell growth. Such a provision ensures a consistent gradient of soluble molecules, nutrients, and drugs. Thereby, spheroid growth is maintained through an optimized synthetic microenvironment.
- Contrary to the above, liquid droplets are continuously generated and manipulated via multiphase incessant flow-driven microfluidics.
- Typical configurations for droplet formation in this manner are flow-focusing and T-junctions (Fig. 19). The flow-focusing regime generates microdroplets by clutching a liquid stream into two immiscible sheath streams, resulting in monodisperse droplets.
- T-junction mode uses a singular sheath flow for bifurcating the dispersed phase into monodispersed droplets. Both provisions use scaffold materials like hydrogel that support cell growth by adding droplets.
- Microfluidic single-phase channels support the 3D cell growth via medium exchange through a perfusion system.
- From a working viewpoint, the fluid is typically passed across a microchannel, akin to in vivo vascularization.

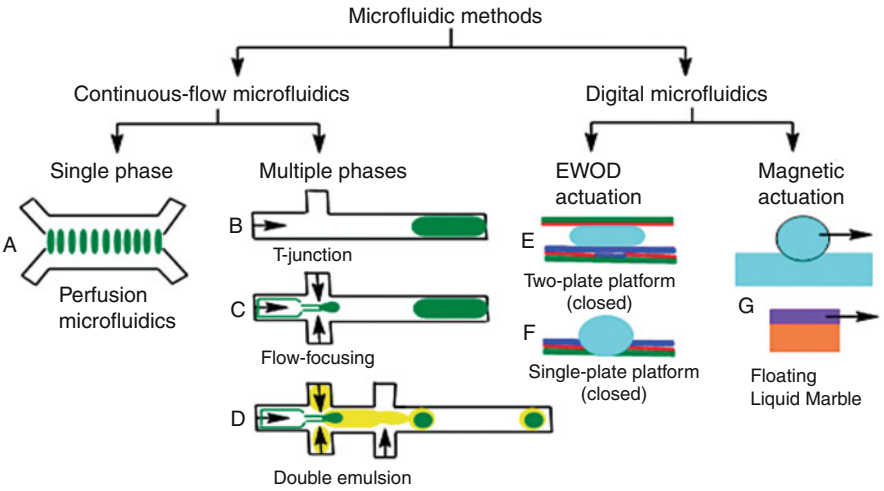

Fig. 19 Chronological steps of the microfluidics-driven cell culture approach, distinguished as continuous and digital flow processes

- Familiar systems for spheroid generation and expansion are restricted to a reactor isolated from its typical surroundings.
- Thereby, spheroid farming is confined to limited duration cultures, manifesting as a debilitating effect on cell viability. This constraint prevails equally well in droplet-driven microfluidics, which does not have a provision for environmental regulation-driven spheroid development.
- To overcome these bottlenecks, *Agastin and colleagues* attempted the growth of multiple tumor spheroid growths using polydimethylsiloxane (PDMS) microbubbles. A physiological flow regime was accomplished inside the microbubble using medium perfusion. This model mimics in vivo avascular tumor conditions.
- Ideally, the perfusion method offers promising mechanisms for anticancer drug testing simply because it not only optimizes the drug exposure but also nutrient and waste exchange.
- In this microfluidic system, media is flown continuously through microchannels and gradually perfuse trapped by cells or spheroids.
- The spheroid development takes place over a long period (e.g., 2 weeks), and the viability of the cells is not affected because of a long-duration culture (Fig. 21).
- Additionally, device fabrication is allowed by this method with the provision of an integrated concentration gradient for high-throughput applications.
- In this method, the perfusion flow is generated by the combination of surface tension force and gravity.
- To increase the throughput, the assay can be scaled up in the 96-well format. *Chen and colleagues* made the initial breakthroughs, in the course of developing an integrated microcapillary network connected with culture medium supply chambers.
- Following these advances, *Sakai and associates* designed an improved microwell array equipped with perfused flows for conducting spheroid-based high-throughput drug testing.
- Another interesting approach involved the merger of multi- and single-phase microfluidics. Spheroids are initially generated in an emulsified droplet, giving rise to coalescence on surfactant droplet (displacement) followed by perfusion exposure.
- Data acquisition and analysis from chemotherapeutic studies revealed higher chemo-resistivity (through perfusion-based format) compared to a static fluidic environment.
- Interestingly, this system also enables the miniaturization of both physiologic and pathologic networks such as angiogenesis and thrombosis in a 3D capillary network. For instance, the platform could act as a splendid scaffold in retaining pathophysiological conditions optimum for tumor angiogenesis and tissue ischemic model (Pamme 2007).

3.5.2 Multiphase Microfluidics

- Over the past few years, numerous review articles have illustrated a comprehensive understanding of the selective formation, arrangement, and manipulation of droplets, in chemistry and biology experiments.

- Of late, droplet-driven microfluidics has emerged as an incentive to enable a microbioreactor-similar working configuration, enabling the growth and characterization of living cells and protocells. Water-in-oil droplets provide ideal environments for culturing cells, allowing continuous aqueous flow breakage into droplets and subsequent encapsulation by a mineral oil-like immiscible phase, paving way for the formation of biocompatible surfactants.
- It is pertinent to note here that immiscible oil droplets are not suitable for culturing multicellular spheroids owing to their obstructive influences on nutrient and gas exchange. So, droplet-based systems allow only short-term cell culturing.
- Nevertheless, to overcome these constraints, a double emulsion system, comprising water-in-oil-in-water constitutional makeup, is still better suited as the droplets act as selective barriers to facilitate the transport of soluble factors and nutrients. The outer aqueous phase maintains needful oxygen permeability although double emulsion morphology frequently generates rigorous polydisperse droplets that are rather easily breakable. A sensitive control of surface wettability is crucial for droplet stability since the droplets also aid in improving heat and mass transfer. These advantages increase the pace of diagnostic results *by* facilitating the conduct of molecular or enzymatic reactions in relatively shorter durations.
- Among several challenges of microbial spheroid culturing using microfluidics, the formation of biocompatible, monodisperse, and stable droplets and the time-dependent cellular activity are the two major concerns. Maintaining droplet stability (a smaller size along with monodispersity) is a critical aspect since culturing of multicellular spheroids requires optimum oxygen, mass, and heat transfer.
- Furthermore, monodispersity is also necessary to ascertain the characteristic impact of a tested drug. Certain primitive parameters affecting the droplet size during emulsification include the typical flow regime (laminar or turbulent), mitigation of shear stress, and the extent of interfacial tension.
- Upon being generated in bulk, shear forces and inertia could squarely contribute to droplet coalescence or bifurcation. To arrest the droplet sizes, surfactants are usually added to the culture medium.
- Surfactants are amphipathic molecules that adsorb at the interface and function by reducing the interfacial tension between the dispersed phase and dispersion medium of an emulsion.
- Though surfactants bring hydrophilic and hydrophobic phases nearby and preserve the homogeneity of forces and medium polarity, the right extent of surfactant addition is critical for the proper functioning of a culture medium as a too high surfactant quantity is likely to be toxic to cultured cells.
- The outer layer of a droplet acts as a selectively permeable membrane that facilitates small molecule transport across this barrier and restores the feasibility of a distinct microenvironment for cell culture. Still, further enhancement in cell growth could be accomplished through encapsulating cells with biological activities.

- Recent interest has drawn specific focus toward the use of hydrogel-laden microfluidic devices for cell culture. A continuous flow microfluidic system can be configured to coencapsulate cells into a hydrogel. In this regard, ***Tumarkin and associates*** have illustrated the use of microgel-based biomaterials for promoting cell functionality in terms of enhanced proliferation and adhesiveness for multiple cell spheroid formation.
- Hydrogels offer highly exploitable structural modulating attributes such as the ability to be cross-linked with chemical residues by chemical stimuli and also with physical processes such as radical reactions, temperature, and photon energy.
- Particles modified into hydrogel provide structural support for spheroid growth and functioning. Capsules supported with hydrogel could be scaled up as spheroid vehicles to facilitate immunological isolation in course of cell transplantation.
- Frequently used modifiable hydrogels for making microcapsules are alginate-PLL (poly-L-lysine APA), thermally responsive hydrogels (agarose, NIPAM-based hydrogel, and gelatin), and photosensitive hydrogels such as polyethylene glycol (PEG).
- Typical gelation methods with characteristic advantages and disadvantages are listed in Table 1, where advantages are specifically associated with optimum cell growth and drug release while the disadvantages substantially comprise the low stability, poor drug release, and the impact on cell viability.
- Several studies have illustrated the use of biomimetic or biodegradable materials as carrier supports useful for cell culture and drug delivery. In one such effort, *Shi and colleagues* have proposed a modified version of the double emulsion solvent evaporation technique to fabricate biodegradable poly (D, L-lactide) porous microspheres, subsequently using them as microcarriers to deliver cells needed for cell-based therapies.
- Another significant contribution reports the application of a double emulsion template to encapsulate droplets transporting multiple biomimetic scaffolds. These scaffolds consisted of porous cores functioning as microcarriers and providing a confined environment for spheroid growth.
- In a more recent development, a reusable device has been proposed to use monodisperse droplets and perform multiple droplet encapsulations. These advances, on the whole, establish a wider significance of multiple encapsulation techniques in a 3D cell-based assay (Günther and Jensen 2006).

3.5.3 Digital Microfluidics

- Continuous flow microfluidic platforms require the pumping and tubing provisions for fluid delivery. As a consequence, handling discrete droplets has certain benefits compared to continuous flow microfluidics.
- Droplet handling could be improved by the introduction of actuation techniques such as magnetic or electrical probes.
- **Digital Microfluidics (DM)** is a specialized domain of microfluidics that facilitates the dual exploitation of microfluidic devices and electrical forces to manipulate discrete droplets. Working culture configurations of DM include closed

Table 1 A summary of different gelation methods used for cell culturing with characteristic advantages and disadvantages of cell growth, handling, and drug delivery

Polymer used	Gelation method	Advantages	Disadvantage
Agarose	Temperature shift	Improved nutrient diffusion and biocompatibility	The operational temperature must support needed cell growth
Gelatin	UV irradiation	Enabling cell-matrix interactions with hydrogel	Combination with hydrogel liquefies
PEG	UV irradiation	Biodegradability	Below par drug release
PEG-PLA	UV irradiation	Optimum delivery of hydrophobic drugs	Vulnerable stability
Alginate	Ion reaction	Long-term culturing through the highly permeable structure	Rapid gelation gives rise to nonspherical particles
Hydrogel matrix	Ion reaction	Increased and improved cell attachment, proliferation, and differentiation	Decreased cell viability
Gelatin + matrigel	Ion reaction	Optimum cell-assembly	Application of matrigel could help induct morphology alteration of cells

PEG polyethylene glycol, *PLA* polylactic acid

(droplets sandwiched between two plates) and open (droplets positioned at the top of a planar surface) modes.

- In the closed format, while the top plate consists of **transparent conductive material**, the bottom plate is **equipped with an array of actuation electrodes.** The surfaces of both plates are coated with a hydrophobic liquid. This will allow friction moderation amid droplet movement.
- Conducting the electrostatic forces through tuning the electric potential of electrodes maintains numerous droplet manipulation activities like dispensing, splitting, merging, and coalescence.
- A combination of **liquid di-electrophoretic** forces (controlled via nonuniform electric field influence on liquid) and **electrowetting** (wetting character of liquid) controls the droplet mobility.
- So, the DM holds manifold advantages over conventional microfluidics like those of low cost, portability, and minimal reagent usage, collectively enabling a faster test result processing. A peculiar advantage of invaluable distinction is the DM's ability to perform multiple biochemical assays at the same time using a planar array of electrodes, enabling the evaluation of multiple real-time test results.
- Indeed, common fluid operation using DM is restricted to a 2D platform, and therefore this methodology suffers from limitations of cross-contamination, solute adsorption, and degradation of soluble factors.
- The 3D transformations were illustrated by submerging droplets in oil between two electrodes. The 3D provision allows simultaneous horizontal and vertical

movement of the droplets, enabling programmable droplets hanging inside the oil.

- This platform has been successfully used to cultivate mouse fibroblasts and NIH-3T3 cells in hydrogel-based discs.
- In due progress, *Au and associates* improved the scaffold-based DM to grow HepG2- and NIH-3T3-cocultured spheroids (organoids) using collagen hydrogels, optimizing the technique to screen hepatotoxicity.
- A further modification has been proposed by *Aijian and colleagues*, illustrating the DM feasibility in enabling a hanging drop-based platform for a spheroid generation. This modification permits liquid handling automation by dispensing the liquid across the connected wells to form hanging droplets. These sequential and reconfigurable manipulations increase throughput for developing spheroid-based assays.
- Another interesting alternative is the **floating liquid marble (LM**) methodology for culturing multiple spheroids of **olfactory ensheathing cells**. This variation involves coating a liquid droplet with hydrophobic powder to form an elastic hydrophobic shell, equipped with fine pores allowing gaseous exchange. Having a low evaporation rate aids in regulating humidity under floating conditions. Furthermore, the floating mechanism allows simpler cell interactions inside the LM aided by an internal fluid flow. Apart from this, LM aids in generating and differentiating embroid bodies. Floating LM locomotion could be controlled via magnetic stimulus, providing a platform for engineering controllable and tunable functions.
- Several modifications have been proposed through magnetic actuation of LM, the feasibility of microcentrifuge-similar functioning using the centrifugal force being one of the major advances. Similarly, the use of magnetism to split LM with lycopodium-iron oxide is also proposed. Thus, it seems quite reasonable that LM could be used to support a DM platform for 3D cellular activities.
- Integration of **microfluidics with optics** lays the foundation for a unique technology, named **optofluidics**. Inceptive optical advances in the domain of microfluidics include the development of optical tweezers and optical vortex.
- Similarly, the application of photoconductivity to the DM has enabled the actuation of droplets via **optoelectronic wetting (OEW)**. The improvement in accuracy of this feature has been attained via photosensitive surfactants with the laser, paving way for 3D droplet manipulations using a single-sided continuous Opto-electro wetting platform.
- Digitalizing and integrating microfluidics with optofluidics, therefore, offers enhanced resolution for biomedical imaging. A significant advance in this direction is the development of a **digital holographic microscope**, allowing lens-free operation besides real-time imaging of transparent PDMS optical-microfluidic channels. The technology has been successfully used for 3D microorganism sensing and automated cell viability detection (Choi et al. 2012; Wang et al. 2017).

3.5.4 Applications of Spheroids in Microfluidics

Organ printing
Organ on chip
Organoid on chip

Organ Printing

- Spheroids present a fine example of organ bioassembly, by their ideal geometry, providing a platform to serve as a "**bionic**" for **bioprinting**. Layer-by-layer (epitaxial) printing of spheroids forms a common methodology for tissue construction in bioprinting. The advancement in computer-aided robotic bioprinting technology allows a precise etching of previously known structures to achieve desired organ/tissue assembly.
- This process owes a sensitive dependence on the specific placement and positioning of dispersed spheroids, allowing controllable fusion in a 3D-grown tissue. The latest advance in this direction is the **Kenzan method,** using microneedles to facilitate spheroid assembly. The process allows micron-level precision to closely link the spheroids. Subsequently, arrays of tightly aligned spheroid form a complex tissue through fusion and synthesize their own **ECM**.
- Tissue construction using spheroids requires a substantial quantity of uniformly sized spheroids, essential for bioprinting with adequate resolution. Scalable spheroid fabrication is the prime requirement to produce the desired quantity of homogeneous spheroids. Ultimately, precise 3D tissue print is achievable through a microfluidic technology-based spheroid biofabricator. The application of droplet-based DM thereby offers a scalable spheroid production technique.
- To improve the applicability of spheroids for bioprinting and tissue fabrication, several other technologies are useful (Fig. 20).
- The best example may be **an electrospun matrix** that can accurately pattern the printed spheroids into desired tissue construct.
- Another example is **magnetic 3D printing.** It is claimed that this technique has the potential to attain precise and rapid 3D tissue construction.
- Another approach made use of magnetic levitation to fabricate tumor spheroids closely mimicking the native microenvironment. Spheroid fusion typically involved 3D cell-cell interaction, critical for larger tissue formation.
- Mechanism of spheroid fusion is rarely attempted, necessitating the quantification of fusion kinetics, via thorough consideration of coalescence time-lapse for two spheroids. Recently, a microfluidic platform has been demonstrated to study spheroid fusion and drug screening, although more sophisticated platforms are still being needed to quantify mechanical aspects (strength and bending strain) of fused spheroids (Lee 2014; Yoo 2015; Xia et al. 2018).

Organ-on-Chip

- Advancements and the need for obtaining enhanced functionalities are creating stiff competition to further the development of lab-on-chip technologies (via cost reduction and efficiency enhancement). The advancements in this technology could reduce the extensive and frequent requirement of animal model testing,

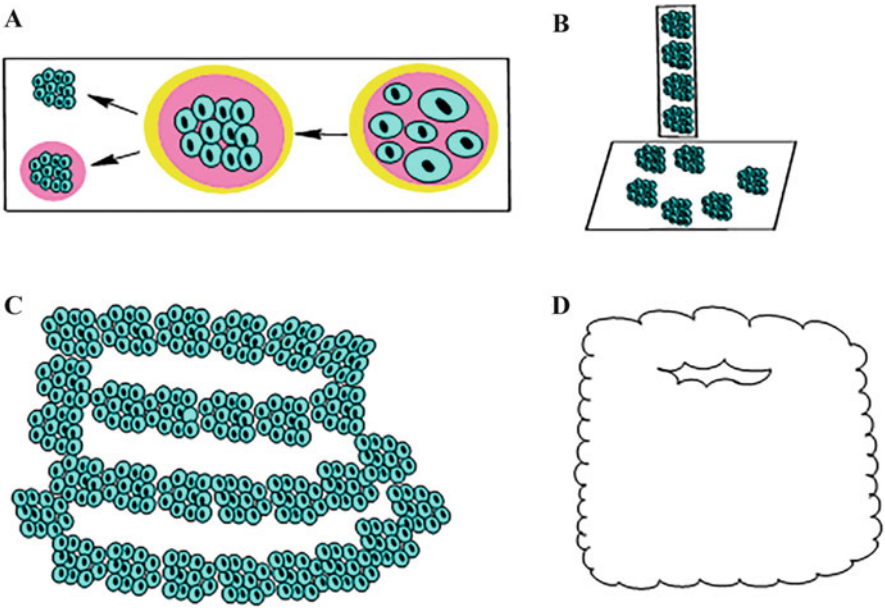

Fig. 20 Schematic presentations of 3D tissue spheroids printing, principles: (**a**) microfluidics-based spheroid fabrication; (**b**) nozzle-assisted spheroid dispensation; (**c**) continuous dispensing forms layer-by-layer tissue spheroid; and (**d**) layer-by-layer tissue spheroid fusion and bioassembly of tubular tissue construct

facilitated through combined advances of fluid physics with 3D cell compartmentalization to propel the organ-on-a-chip device popularity to the next level. This technique simplifies clinical bioanalysis by integrating realistic organ replication in a single device.

To incept the organ on a chip, the liver spheroid and neurosphere cultures are cultured separately. Then these two cultures are connected through the microfluidic circuit.

- To fabricate the scaled-up organ-on-a-chip, spheroid combination can be done which ultimately resulted in spheroids fusion and tissue formation. These tissues may serve as a model to simplify the physiological function of an organ.
- Advanced features of organ-on-a-chip are attained by the integration of working assembly with microsensors, capable of detecting cell and environmental cues. A frequent stimulus detected through such improvements is the transmembrane electrical resistance across cell barriers to detecting cell migration. Efforts are in swift progress for biochemical and biophysical analysis to ascertain adequate extents of tissue mechanics, invasion, and fusion. Such traits of this technology are well known to facilitate qualitative inspection via molecular diagnostic probes (working through detection of DNA, proteins, enzymes, and several other biological sources).

- Biological events described by the interactions between two or more organs could be accurately tracked, via *the* organ-on-a-chip model. This probe has an inbuilt database for the interactions of multiple organs in a physiologically replicating environment. Advances like these could revolutionize the ascertainment of drug metabolism and toxicity to translate basic bench research into clinical practice. Fabrication of this device could be done via using spheroids to replicate the in vivo cellular conditions and enhance the results.
- Thereby, the ability to screen the responses of multiple organ systems using a single device is conferred the terminology, "**body-on-a-chip.**" The model is equipped with a fluid stream, acting as a network of surrogate blood vessels to facilitate biochemical interconnection among all tissue compartments, allowing the evaluation of drug effects via multiple physiological interactions. Through such abilities, the platform endows significant promise and may further improve the detection efficacy for scrutinizing novel drug candidates. Features of this developed assay advance the analysis of pharmacokinetic and pharmacodynamics aspects to predict drug safety. Figure 21 (above) describes a schematic description of the possible use of spheroids to fabricate multiple organ-on-a-chip provisions (Huh et al. 2011).

Organoids on Chip

The quality of tissue function by organized spheroids is rather limited. As a consequence, in recent years developments for the utilization of organoid cultures have gained attention.

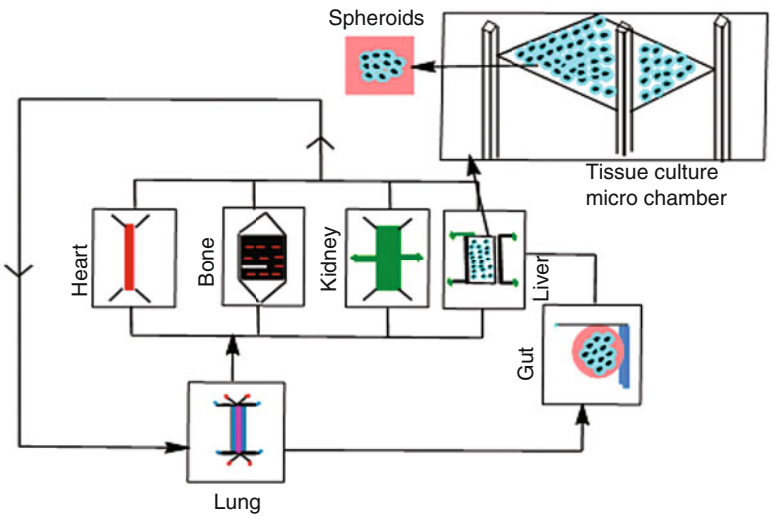

Fig. 21 Pictorial description of spheroid integration with microfluidic-based multiple organ-on-a-chip models

Using totipotent embryonic stem cells, organoids are generated. The process belongs to the gradual aggregation and assembly of stem cells into a spatially patterned structure-supporting organogenesis. Organoids could also be developed using induced pluripotent stem (IPS) cells, derived from a patient representing a personalized cell system functioning as an individual-specific disease model.

Organoid cultures could be replicated on the microfluidic platform to model distinctive organ features, comprising development, homeostasis, and diseases. These cultures ultimately give rise to a higher order of tissue organization, such as a hollow sphere.

- Often, organoids share many features in commonality with the physiological models for drug metabolism and toxicity evaluation. For instance, organoid development for the gastrointestinal system is a significant resource for drug analysis experimental models. The inceptive stage of intestinal organoids was conceptualized using stem cells encoding leucine-abundant repeats composed of G-protein-coupled receptor 5 (LGR5). These LGR5 stem cells are capable of differentiating into gastrointestinal system organs, including the hepatic system. Subsequent progress of the hepatic system aids in screening drug safety via analysis of drugs and toxin biotransformation. Emerging attention to designing dynamic microfluidic systems could aid significantly in reducing the dimensions of hepatic organoids via a single chip-facilitated high throughput screening. For instance, the DM-enabled drug analysis was optimized via liver organoid arrays, more familiar as "General Organoid Droplet Exchange Procedure (GODEP), enabling reagent exchange mediated fluid control within the liver organoid." The microfluidic systems necessitate a continuous media circulation, in light of hurdles encountered amid the generation of growth factors, cell signals, and drug gradients. Still, maintaining the organoid position in a continuous flow microfluidic system indeed presents a challenge to chemical engineers.
- This strategy has been recently in active consideration using encapsulation and subsequent loading of liver organoids in perfused C-shaped trap arrays. This approach sustains a hepatic tissue position during exposure to diverse fluid flow rates. Thus, microfluidics-assisted organoid encapsulation provides a prominent role in therapeutic delivery systems. A befitting example in this regard is the LSFM4LIFE project, an ongoing EU Horizon 2020 program that intends to achieve cellular therapy for type 1 diabetes using Human Pancreatic Organoids (HPOs). Further, alginate-poly-L-lysine is used to encapsulate cells for the secretion of antibodies and therapeutic proteins. The microcapsules developed thereof are termed immunotherapeutic organoids, which could be implanted in vivo for therapeutic intent.
- Such materials prevail as extraordinary substitutes for conventional drug delivery to treat cancers and immunological disorders. The amalgamation of biomaterials in these devices further advances the organoid-comprising platforms. For instance, prototyping hyaluronic acid (HA) as the substrate is required to design 3D contractile organoids of cardiac origin. Topical investigations, herein, demonstrate the formation of functional cardiac organoids

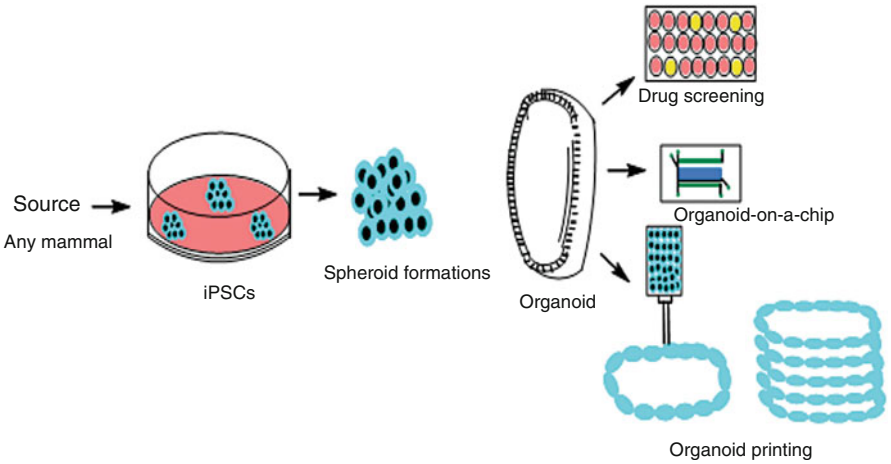

Fig. 22 Organoid development and integration with microfluidic technology for drug screening, organoid-on-a-chip development, and organoid-driven printing

through the 3D print of spheroids. Besides this, hepatic organoids configured via assembling multiple spheroids using priorly fabricated cavity optimized through acoustically driven nodes have been demonstrated. Similarly, the formation of human airway epithelial cells using epithelial organoids for countering the cues residing in an extracellular matrix as a nano-dimensional patterned substrate is also well demonstrated. Figure 22 summarizes the potential of induced pluripotent stem cell-derived organoids (Baker 2011; Sunghee Estelle Park et al. 2019).

4 Conclusion

Mammalian cell culture in flat 2D culture vessels (Petri plates/flasks) is considerably different from in vivo architecture of mammalian cells/tissues and organs. Therefore, mammalian cell culture in 3D platforms has been developed which closely mimics the in vivo environment of mammalian cellular physiology. To create a 3D platform, it is highly essential to understand the actual configuration and the various constituents of extracellular matrix (**ECM**) that coordinate with the mammalian cells to form tissues and organs. For 3D culture, five different platforms such as **scaffolds, scaffold-free spheroids, gels, bioreactors,** and **microchips** are used. These platforms are made up of natural materials such as **collagen, fibronectin, gelatin, laminin, and vitronectin,** or synthetic materials such as **agarose,** all of which are also prevalent in mammalian **ECM.** A variety of techniques, such as **3D printing, particulate leaching, electrospinning,** etc. are utilized to prepare various 3D platforms. This chapter describes the various platforms developed for the 3D culture of mammalian cells, their specific architecture, and the various materials needed to

synthesize all these platforms along with various techniques involved. The mammalian 3D cell culture technology is useful in the **research laboratory to understand the physiology of various cells.** It is also useful in **clinical settings (such as in cancer biology)** and **regenerative medicine for organ and organoid developments.**

5 Cross-References

- ▶ Large-Scale Culture of Mammalian Cells for Various Industrial Purposes
- ▶ Mammalian Cells, Tissues and Organ Culture: Applications
- ▶ Organ, Histotypic and Organotypic Culture, and Tissue Engineering

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Organ, Histotypic and Organotypic Culture, and Tissue Engineering

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_14-2

Abstract

Organ, histotypic, and **organotypic** culture using mammalian three-dimensional (3D) cell culture technologies are the primary focus of this chapter. The major purpose of organ, histotypic, and organotypic culture is to create in vitro models comparable to the in vivo environment which may replace old, damaged/injured, or diseased organs. **Organ culture** describes the in vitro maintenance of growth of a part or whole of an organ in which the various tissue components, such as parenchyma and stroma and their anatomical relationship and function(s), are preserved. The methods utilized for organ cultures are ***plasma clots, agar gels, raft, and grid***. The histotypic tissue culture allows cells to be grown in high densities in a 3D matrix or scaffolds, thereby creating in vitro morphologies that closely mimic the realistic in vivo tissue functioning. In organotypic culture, a component of an organ is created using cells from different lineages in proper ratio and spatial relationship under laboratory conditions. Additionally, this chapter describes **tissue engineering**, an in vitro process that produces entirely functional tissue (closely resembling the natural tissue) by seeding cells into a biomaterial matrix called a **scaffold**. The scaffolds are made up of either natural biomatrix materials or synthetic materials. The general purpose of tissue engineering is to implant the generated tissue into the body's replacement of old, damaged/injured, or diseased tissue or organs. Besides the general process of tissue engineering, the benefits and shortcomings of tissue engineering are also included in this chapter.

Keywords

Organ culture · Histotypic culture · Organotypic culture · Plasma clot method/watch glass method · Agar gel method · Raft method · Grid method · Tissue engineering · Scaffolds · Regenerative medicine

1 Introduction

This chapter describes an overview and importance **of organ, histotypic, and organotypic cultures**. In a mammalian body, an organ is defined as a specialized structure in a body that is capable to fulfill specialized function(s). Basically, in an organ, various tissues are collectively involved in maintaining the overall structural and functional integrity. The culture of a whole or part of an organ is called **organ culture**. While in cell culture a particular type of isolated cell or a mixture of isolated cells is cultured, in organ culture, either whole or a part of an organ is cultured. Thus, in organ culture, isolation/separation or disaggregation of tissues/organs into cells is not

witnessed. Moreover, cellular differentiation can be monitored in organ culture. It appears that embryonic organ culture is an easier alternative to organ culture derived from adult mammals. Greater oxygen requirement by the adult organ cells may be one of the reasons for their slow growth. The **support material**s used in organ culture comprise filter-well inserts made up of **ceramic, collagen** or **nitrocellulose, stainless steel grid, semisolid agar gel, clotted plasma, micropore filter, lens paper, or strips of perspex/ plexiglass**. Four different organ culture methods are described here, namely, (i) **Plasma clot method, (ii) Agar gel method, (iii) Raft method, and (iv) Grid method**.

For the histotypic culture, (A) Gel and sponge method, (B) Hollow fiber method, (C) Spheroids method, and (D) Multicellular tumor spheroid method are important. ***In histotypic culture, cell lines are grown in high density in a three-dimensional (3D) matrix. On the other hand, in organotypic culture, cells of different lineages in a proper ratio are cultured in a laboratory condition to create a component of an organ.***

The major purpose of organ, histotypic, and organotypic cultures is to create in vitro models comparable to the in vivo system. The words **tissue engineering** and **regenerative medicine** are complementary to each other. Scientists from various **institutes of regenerative medicine as well as tissue engineering** believe that, 1 day, they will be able to grow complete structures of various organs, useful for human transplantation. In summary, this chapter enhances the reader's overall knowledge of organs, the histotypic and organotypic cultures.

2 The Concept of Organ, the Histotypic, and Organotypic Cultures

- **An organ** is defined as a particular specialized structure within a mammalian body, capable of fulfilling specialized function(s).
- Each organ serves as a separate yet integrative entity in maintaining the overall structural and functional integrity of a mammalian body.
- The various tissues within an organ are collectively involved in maintaining its overall structural integrity alongside the characteristic function(s).
- **Differentiation at the cellular level** has mostly been studied in organ, rather than cell, cultures.
- ***In organ culture, the cell-to-cell interaction and formation of a structure closely resemble the* in vivo *structure, and therefore cells are integrated as a single unit.***
- ***In organ culture, due to the generation of integrated into vivo-like structure, it is capable of cell-to-cell interaction through adhesion and integrin molecules as well as bidirectional (inside out and outside in) cell signaling.***
- By definition, **organ culture** describes the in vitro growth maintenance of a part or whole of an organ, wherein various tissue components such as parenchyma, stroma, and their anatomical relationship and function(s) are preserved.

- Characteristically in an in vitro organ culture, the **explanted tissues** closely resemble the in vivo parental tissues, retaining their parental ability to allow architecture differentiation or preservation and organ functioning.
- Of note, **an explant** is a cell, organ, or piece of tissue, transferred from animals or plants to a nutrient medium for culture and growth.
- By selecting the suitable culture conditions, the **outgrowth of isolated cells from the periphery of the explant is discouraged and minimized**.
- For histotypic culture, the cell lines should be grown in three-dimensional matrices to a high density.
- In an organotypic culture, various cells having different lineages are cultured in a proper ratio that resembles the in vivo conditions. This produces an **organ component**.
- Organ, histotypic, and organotypic culture methods can be analyzed via **histology, autoradiography**, and **immunochemistry**.
- Together organ, histotypic, and organotypic culture techniques enhance our understanding of the development/growth and functional behavior of organs.
- The organ created may replace the diseased/injured/damaged organs and therefore may be of significant value to **Institutes of Regenerative Medicines**, as well as **tissue engineering** (Fell 1953; Borghese 1958; Kahn 1958; Merchant et al. 1964).

3 Organ Culture: Brief History of Mammalian Organ Culture

- In 1897, **Loeb** first attempted the organ culture.
- Loeb generated for the first time a **plasma clot** by mixing 15 drops of plasma with 05 drops of embryo extract.
- **NB:** All the initial studies used chick plasma and chick embryo extract.
- He took the plasma clot in the test tube and incubated the adult rabbit ovary, thyroid, liver, and kidney and observed that these organs retained their normal histological features for up to 3 days.
- In the year 1910, a scientist named ***Burrow*** for the first time established the **chick embryo** organ culture outside the body. Burrow used a technique, famously named as "**Plasma Clot Method**."
- Later in 1919, ***Loeb*** *and* ***Fleischer*** reported that to prevent central necrosis of the cultured explants, it was necessary to fill the tube with O_2.
- Pioneering works of organ culture technology were reported in 1949 from ***Hardy*** during the growth of hair and hair follicles.
- In 1963, ***Cleffman*** studied the pigment formation in the hair-follicle melanocytes of agouti mice.
- Under organ culture, almost every organ of the mouse has been cultivated in vitro.
- In organ culture, the whole organs or small fragments of an organ, with their special and intrinsic properties intact, are used for culturing.

- The adult human organs require more oxygen than their embryonic counterpart, and this may be one of the reasons for the difficulty to culture adult human organs or their parts.
- This is also one of the reasons for the preferential culture of embryonic organs, rather than adult organs.
- The specially designed apparatus are used to culture several adult organs using a special medium. The most preferred apparatus is ***Towell's II culture chamber***.
- Since **serum was found as toxic**, serum-free medium (**e.g., T8**) was used, with the special apparatus (e.g., ***Towell's II culture chamber***) permitting the use of 95% oxygen.
- The most important original references in the development of organ culture include the publications from Fell (1953), Borghese (1958), Kahn (1958), and Merchant et al. (1964).

NB: Embryonic organs are easier and faster to grow in the artificial organ culture environment.

4 Factors Affecting Mammalian Organ Culture

Several factors affect the successful culture and growth of organs. These factors must be optimized and maintained for successful organ culture.

1. Gas and liquid phase
2. Structural integrity
3. Blocked differentiation

Here is a sequential presentation of the above factors that affect the organ culture:

4.1 Gas and Liquid Phase

Diffusion of gases and nutrients into the cultured organs may be an issue in successful organ culture. If the organs are considered a solid mass, it would be difficult to exchange gases and nutrients across these solid masses. However, if the cells grow as monolayers there will be an easy transfer of gases, nutrients, etc. Under these conditions, it is necessary to transport the exact amount of gases and nutrients into the cultured cells. The **liquid-gas interface incubation of the tissues/organs** is necessary for the proper transfer of gases and nutrients.

While incubating in a liquid-gas interface, the following points should be kept in mind:

- The cells occupy a special shape when growing in a liquid-gas interface at the appropriate depth.

- The diffusion of gases does not happen if culturing is done at a deeper level of the medium/liquid.
- As and when the tissues/organs are cultured in the shallow medium, the outgrowth of the cells would occur due to more surface tension. In this case, no accuracy of the culture can be maintained.
- For ideal growth, O_2 is supplied either as hyperbaric O_2 or as 95% pure O_2.

NB: The limited diffusion of extracellular molecules into thick tissues restricts organ culture to embryonic or thin organs.

4.2 Structural Integrity

Cells in an organ culture must interact in a manner so that the combined effect of cells is the same as an integrated organ, and thus intercellular communication could be sustained.

4.3 Blocking of Differentiation

In general, the induction of differentiation of the cultured tissues and organs may result in a loss of their proper regulation. This may be one of the major reasons for blocking differentiation during tissue/organ culture.

4.4 Support Materials Needed for Organ Culture

- The organ culture mandates optimization of nutrient and gas exchanges by keeping the tissues at the gas-limited interface.

The support materials used in organ culture are as follows:

- Semisolid agar gel
- Clotted plasma
- Micropore filter
- Lens paper
- Perspex or plexiglass strips
- Stainless steel grid
- Ceramic, collagen, or nitrocellulose-made filter well insert

The successful use of these inserts leads to the development of functionally integrated various epithelia such as renal epithelium, stratified epidermis, integrated thyroid epithelium, and intestinal epithelium.

5 Sources of Organ Culture

- Developing chick or any other animal's embryo.
- Organs would be isolated from adult animals.
- Human organs/tissues (e.g., skin, kidney, cornea, etc.) from a voluntary donor.
- Surgically removed organs of a disease-affected person, intended for transplantation replacement.
- Human organs would be isolated from an accidentally dead person.
- Aborted human fetus.

NB: For using human subjects, the suitable ethical committee must permit the work conduct even before taking the consent of the suitable organ donor. Similarly, one must take the permission of the animal ethics committee before sacrificing or even collecting any animal.

6 General Requisition for Organ Culture

A researcher must complete the following steps before commencing the organ culture

- Complete all the formalities and maintain all the ethical standards before sacrificing animal embryos or adult animals.
- Similarly, all the ethical norms including the donor's signed approval, and approval of human organ isolation protocol by a constitutional departmental or institution committee, are necessary before even collecting any organ.
- The organs to be cultured must be fresh and cultured as soon as possible (within 1–3 h).

NB: Generally frozen or stored organs do not grow and therefore only fresh organs are recommended for culturing.

- Organs **must not be dried** before culture. Drying results in loss of growth capacity.
- Thus, immediately after collection, the organ must be stored in Hanks Balanced Salt Solution (**HBSS**) or in suitable other salt solution (DPBS/PBS) until being used for culture.
- Remove the organ from the primary source (e.g., chick embryo/other animal organs, etc.) using a dissection microscope.
- For humans, only suitable expert professional physicians or technicians are allowed for organ collection.
- During dissection, the organs must be kept moist with HBSS containing 1–2% serum. Dissection must be performed carefully without damaging the tissues within the organ.
- Now transfer the dissected properly sized **(generally <1 mm)** tissue piece from the organ in a cavity slide containing HBSS for its careful washing.
- Now choose **a particular organ culture technique** and proceed with the culture procedure.

- In general, the tissue should be cultured at the **gas-medium (liquid) interface**.
- Once the tissue is placed, incubate the culture vessel in a CO_2 incubator, with 95% moisture at 37 °C.
- Change the medium (M199 or CMRL1066, for human organ culture) as frequently as desired.

NB: M199 is a general medium used for various organ cultures.

However, a specific organ or outgrowth such as cancer tissue may need a specialized medium for its growth. For example, a section of the breast cancer tissue may grow well in MEM or DMEM medium.

Four techniques are generally used to analyze the cultured organs:

Microscopy for live growing organs
Histology
Autoradiography
Immunohistochemistry

7 Methodologies of Organ Culture

The following four methods are used for organ culture:

1. Organ culture by plasma clot method
2. Organ culture by agar gel method
3. Organ culture by raft method
4. Organ culture by grid method

There are pros and cons of each of the above cultural techniques. Ahead is a brief discussion about them.

7.1 Organ Culture by Plasma Clot Method

- ***Fell*** and ***Robinson*** introduced this method to culture the avian limb bone rudiment development.
- Later on, this method was utilized not only for the growth and differentiation of other avian organs but also for the development of mammalian organs.
- Before culture, the chick plasma and chick embryo extract are prepared.
- Now, the chick plasma and chick embryo extract are placed on the surface of a clot that is put in a watch glass. Therefore, this procedure is also famously called as "***Watch Glass Technique***."
- A glass lid sealed with paraffin wax may or may not close the watch glass.
- In a Petri dish carpeted with moist cotton wool or filter paper, one or two such watch glasses are enclosed.

This method requires chick plasma and chick embryo extract. So, before starting the procedure one must prepare the chick embryo extract.

7.1.1 Preparation of Chick Embryo Extracts

Chick embryo extracts are one of the common materials needed for various organ culture methods.

The materials and methods needed to prepare chick embryo extracts are as follows:

Materials for Chick Embryo Extract Preparation

- Fertilized hen eggs incubated for 7–8 days
- Petri dishes
- Straight forceps
- Curved forceps
- Curved scissors
- Homogenizer
- Glass with teflon pestle
- Centrifuge tubes (15 and 50 ml)
- Pasteur pipettes
- Wide-mouthed pipettes, made via cutting the tops of ordinary pipettes
- Storage vials
- Hanks balanced salt solution (**HBSS**)
- 70% Alcohol

NB: All reagents and instruments must be sterile.

Method to Prepare Chick Embryo Extract

The following lines describe the preparation of chick embryo extracts:

- Soak cotton wool with 70% ethanol.
- Wipe the blunt end of the egg and sterilize the surface.
- Tapping the upper end of the shell, crack it and peel inward.
- Then tear open the inner shell membrane covering the embryo with a pair of straight forceps.
- Use a pair of curved forceps, lift the embryos out by their heads, and then gently drop the embryos into a Petri dish.
- Wash the embryo with HBSS.
- Remove the eyes.
- Use a pair of curved scissors to mince the embryos by hand.
- Transfer the minced tissues to a **homogenizer**.
- Hand grind the minced tissue for a few minutes.
- Add an equal amount of HBSS to homogenate and mix it.
- Centrifuge the mixture at **20 g for 10 min at 4 °C**.
- Remove the supernatant.
- The final supernatant should be free of cells and appear opalescent.

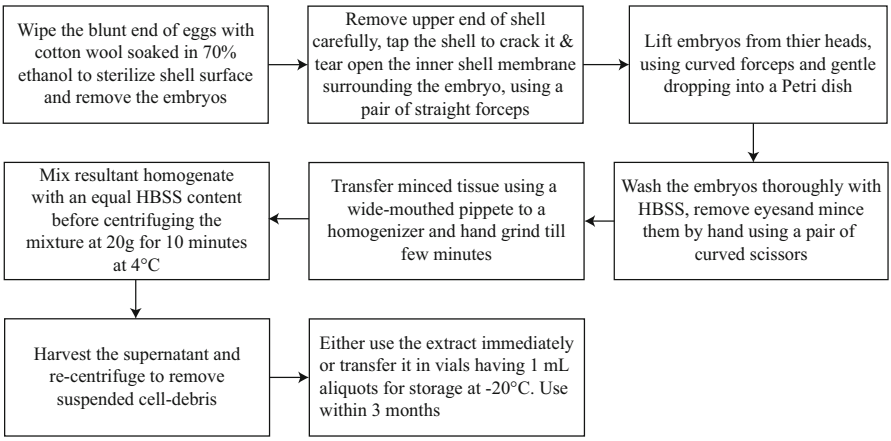

Fig. 1 Flowchart summarizing the preparation for chick embryo extracts

- The extract can be used immediately or transferred to vials in 1 ml aliquots for storage at −20 °C.
- Use within 3 months. Figure 1 summarizes the steps for an easy follow-up.

7.1.2 The Plasma Clot Method of Organ Culture

The plasma clot method has the following steps:

- Take a watch glass and add 15 drops of chick plasma and 5 drops of chick embryo extract and mix them.
- Take a Petri dish, put a cotton wool pad, and moisten it.
- On the top of the moist wool, put the watch glass.
- The cotton wool would wait as long as the culture continues.
- Now carefully transfer the washed dissected tissue pieces **(1 mm** or **less, thin slices grow better because of easy nutrients and oxygen transfer)** on the top of plasma clots in the watch glass.
- Place the watch glass in a CO_2 incubator at 37 °C **(Although, in general, incubation time is up to 4 weeks, it may vary)**.
- Allow the organ to grow (Fig. 2).

7.1.3 Advantages of Plasma Clot Method

- Inexpensive
- Permits light microscope, hence suitable to study hair growth, fetal mouse skin differentiation, etc.
- Easy to follow technique

7.1.4 Disadvantages of Plasma Clot Method

- Clot liquefies in the vicinity of explants, consequently immersing them in the medium.

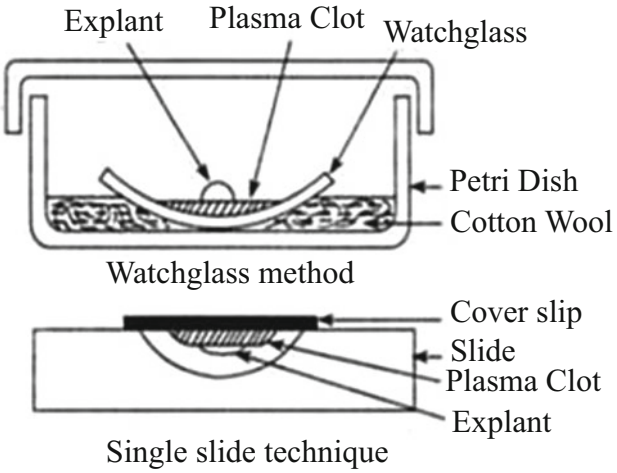

Fig. 2 Schematic representation of Plasma Clot method

- Short culture duration (**<4 weeks**).
- The complexity of the medium forbids biochemical inspection.

7.1.5 Application of Plasma Clot Method

- The major use of the plasma clot method is to study embryonic morphogenesis.
- The other use of this method is to study the effects of carcinogens, vitamins, hormones, growth factors, etc. on cultured tissues isolated from adult organs.

7.2 Organ Culture by Using the Agar Gel Method

- ***Spratt*** introduced the **agar gel method**.
- Later on, ***Gaillard's technique*** was modified by ***Wolff and Haffen*** and used agar gel contained in an embryological watch glass.
- The major use of this technique is morphogenetic and in developmental studies.

7.2.1 Organ Culture by Agar Gel Method Has the Following Steps

- Make an organ culture medium by mixing three parts of **chick embryo extracts with** three parts of **horse serum**.
- Add seven parts of 1% agar in HBSS into the above medium to assist in solidification.
- ***So, the agar gel is prepared by mixing chick embryo extracts: horse serum with 1% agar in HBSS at 3:3:7 stoichiometries.***
- The agar-containing medium is a semisolid material; it does not liquefy. Therefore, it provides mechanical support for the cultured organ(s).

- Take two cataract knives, and with the help of them transfer the organ to the agar gel. Alternatively, transfer via sucking them up in a wide-mouthed pipette with HBSS, and finally depositing them on the agar.
- Use two needles to orient the explants on the agar.
- Use a fine pipette to suck off the excess fluid.
- Usually, each gel accommodates one explant.
- ***"The optimum size of the explant is (1.5 × 1.5 × 1) mm, with the maximum size being (2 × 2 × 1.5) mm. However, if the tissue is very thin (<0.5 rams thick), the other dimensions can be larger."***
- To seal the glass lids onto the chambers, take a small paint brush and apply at least two warm paraffin wax (60 °C) coats.
- Now transfer the chamber to the CO_2 incubator and incubate at 37 °C.
- **NB: Instead of putting the chamber directly on the metal shelves of the incubator, it should be put using polystyrene foam tile. This will insulate the chamber from the metal tiles.**
- Figure 3 summarizes the steps for an easier follow-up.

7.2.2 Note the Following

- While embryonic tissues/organs grow very well using agar, the adult organs do not survive in this medium. ***This may be related to the more oxygen requirement of the adult tissues.***

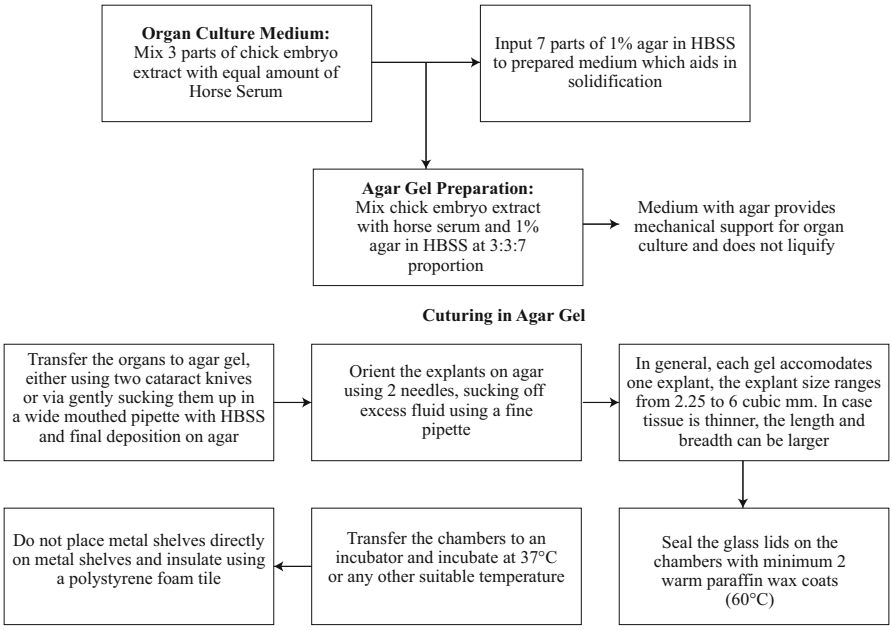

Fig. 3 Quick-to-follow summary of organ culture using the Agar Gel method

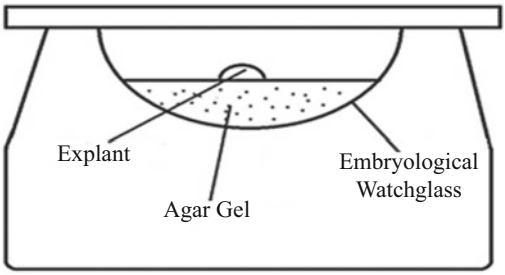

Fig. 4 Agar gel in embryological watch glass

- Some of the experimental observations such as morphogenetic changes and cytodifferentiation require only 3–7 days of culture. These experiments are completed within 3–7 days and do not require any changes in the cell culture medium.
- In case of longer duration experiments, the explants mandate a relocation to a fresh agar gel every 5–7 days. In this case, fresh chambers are used to make a fresh gel with a defined medium and the same proportion of agar as used before.
- To transfer the explants from the old gel to the new gel, first remove the lids from the old watch glass using a warm razor. Now take two cataract knives or one cataract knife and a dissecting needle and lift off the explants from the old gel. Wash the explants in HBSS containing a cavity slide. Transfer the washed explants to fresh gel. Finally, using a small paint brush and paraffin wax seal the watch glasses with a fresh sterile glass lid (Fig. 4).
- For examining the viability of the cultured explants, they can be viewed using daylight or via a light source from the dissecting binocular. While the translucent and shiny surface tissues indicate good health of the cultured explanted tissues, opacity suggests loss of viability or the necrosis initiation of the explanted tissue.

7.3 Organ Culture by Raft Method

- The initial works were carried out by ***Chen*** *and associates*. They used cleaning microscope lenses (Gurr) that are nonwettable and float on the fluid medium. A **25 × 25 mm raft of lens paper** which was floated on serum in a watch glass was used by Chen et al. to put four to five explant cultures.
- It was ***Richter*** who improved the floating properties of this methodology via lens paper treatment with silicone.
- Further modification of the lens paper was done by ***Lash*** **and accomplices,** who combined the **lens paper** with the **Millipore filters**.
- In the process, the Millipore filter covers a small hole that was punched in the center of the lens paper.
- It was claimed that either side of the filters is suitable for the culture of different cells.

- It was ***Shaffer*** who replaced the lens paper with **rayon acetate**.
- Further modifications of the rayon acetate strips were done by several scientists to make them float on the cell culture medium.
- For this process, the four corners of the **rayon acetate strips** were treated with silicone.
- Since the rayon acetate is easily soluble in acetone, it can be dissolved during the histological procedures via acetone immersion.

7.3.1 Organ Culture by Raft Method Has the Following Steps

- Take a watch glass.
- Put serum into the watch glass.
- Float **a raft of lens paper or rayon acetate on the serum**. Rayon acetate rafts are made to float on the serum via silicone treatment of their corners.
- Similarly, the flowability of lens paper gets enhanced on silicone treatment. On each raft, usually, four or more explants are placed.
- A combination of clot and raft techniques can be utilized for explants culture. To offer the combination, the explants are first placed on a suitable raft before their placement on a plasma clot.
- The medium changes will be easy because of this modification, and it also prevents the explants from sinking into liquefied plasma.

7.3.2 Major Drawback of the Raft Method

The floating of a raft on a fluid medium does not provide an ideal condition since the raft often sank with the tissue in varying medium depths. Thus, the main objective of the raft method, i.e., floating on the medium with the tissue cannot be fulfilled (Fig. 5).

7.4 Organ Culture by Grid Method

- In 1954, the grid method was introduced by ***Trowell***.
- The name indicates the use of metal grids, instead of the raft which overcomes the difficulties faced by the raft method.
- Initially, tantalum wire gauze was used to make the grid.

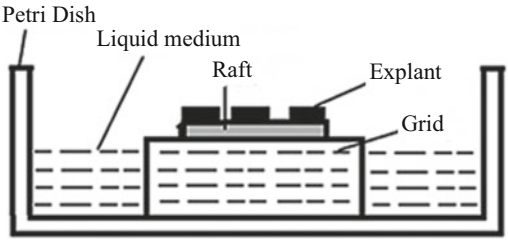

Fig. 5 Grid technique for organ culture

- Later on, stainless steel or titanium was used that made a continuous, expanded, rigid raft.
- In the original culture method, Trowell used adult mammalian tissues/organs and perfused the tissue with carbon dioxide and oxygen.
- He was able to preserve the histological structure as well as the viability of various adult human tissues such as kidneys, prostate glands, pituitary gland, and thyroid gland.
- At present, the technique has been simplified (particularly the gas phase) and is still in use.

7.4.1 Steps in Organ Culture Using Grid Method

- To prepare a grid, **25 × 25 sq.mm pieces of a suitable wire mesh or perforated stainless steel sheet are used**. Further, the edges of this sheet are bent to form four legs of about 4 mm height (Fig. 6).
- The placing of the tissues on the grid is completed either by directly keeping the tissues on the grid or if the tissues are soft, first they are put on a raft, and then the raft is kept on the grid.
- Now the culture chambers are used to put the gird into them.
- In the next step, the culture chambers are filled with the medium up to the GRID level.
- The chambers are infused with both oxygen and carbon dioxide to meet the demand of the adult tissues (the embryonic tissues do not need the infusion of oxygen and carbon dioxide).

7.4.2 The Advantages and Disadvantages of the Grid Method

NB: It is of fundamental interest to understand how cells build tissues and organs. However, the long timescale of mammalian development and its location deep within an opaque animal restrict most organogenesis investigations to comparisons of fixed sections from different animals.

- Most of the three-dimensional (3D) cultures were initially developed for direct observation of the development process.

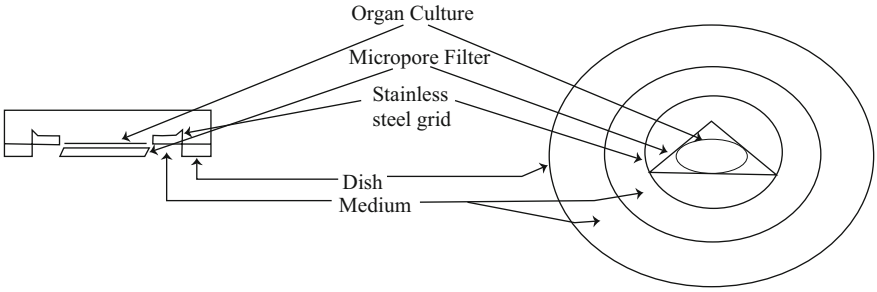

Fig. 6 Schematic representation of organ culture being performed on a stainless steel grid

- The mechanism of organ formation is difficult to understand due to the use of a large number of heterogenic cells with diversified morphology and functions.
- Due to a lack of understanding of the development trajectory, it is difficult to understand the mechanistic implications of mutant phenotypes.

Advantages of Grid Method

- The method allows separate handling of skeletal and soft tissues, wherein the former are directly placed on the grid but softer tissues like glands or skin are initially put on rafts and thereafter transferred to the grids. This provision preserves the tissue-specific viability for a long duration and helps to systemize the monitored response.
- To meet the high oxygen requirement of adult mammalian organs, the grids are placed in a culture chamber to which O_2 and CO_2 are provided.
- Several modifications of the standardized grid method are primed for studying the development and differentiation of adult and embryonic tissues.

Disadvantages of Grid Method for Organ Culture

- Mandates handling by skilled manpower.
- Costly method due to high dependence on culturing conditions.
- There is no provision to simultaneously culture the organs from two distinct tissues, often encountered with contrasting growth and development prospects.

8 Histotypic Cultures of Cells/Tissues

- The histotypic tissue culture allows the cells to be grown in high densities in 3D matrix or scaffolds, thereby creating in vitro morphologies closely mimicking realistic tissue functions.
- In this procedure, only single cells are grown over 3D scaffolds.
- Using the histotypic culture, it is possible to use **dispersed monolayers to regenerate tissue-like structures**.
- The most important aspect of the histotypic culture is the growth and propagation of cell lines in the 3D matrix to yield a high cell density (Maslow and Mayhew 1974; Caffe et al. 1989).

8.1 Method of the Histotypic Culture

The basic material needed for the histotypic cell culture is the primary cells, directly isolated from a fresh organ. The culture should enable the creation of a matrix or scaffold conferring the cells a 3D architecture, resembling the natural extracellular matrix (ECM). Other materials needed for the histotypic culture are general requirements such as medium, CO_2 incubator, etc.

The chronological steps in the histotypic culture are as follows:

1. Isolation of cells from organs
2. Creation of a 3D scaffold
3. Droplet/cell suspension technique to seed the cells into a 3D scaffold
4. Incubation and culture of cells in scaffold
5. Use of the cultured scaffolds
6. Cautions and assets of the histotypic culture practices

Here is a brief discussion of the above steps:

8.1.1 Isolation of Cells from Organs

- Sterilization of the work area with 70% ethanol.
- Autoclave/sterilize the dissecting instruments.
- UV sterilize and clean the biosafety cabinet with 70% ethanol.
- Place a sterile drape on the work surface.
- Then put the sterile surgical instruments on the sterile drape.
- Open a sterile number 20 scalpel blade.
- Euthanize the specimen.
- When ready, put the specimen in a surgical tray.
- Aseptically isolate the specific organ(s) from the specimen.
- Organs can be collected from the experimental animals or discarded lots from the transplanted surgery or accidental death-meeting persons after fulfilling all the ethical rules and regulations.
- Once the fresh organ is collected, it should be soon subjected to cell isolation either via **proteolytic digestion or mechanical disintegration**.
- However, proteolytic digestion is the most widely used procedure, the details of which have already been discussed in various chapters including chapter ▶ "Isolation and Primary Culture of Various Mammalian Cells," and therefore are not described here.

8.1.2 Creation of a Three-Dimensional Scaffold

For 3D culture, designing a scaffold is necessary on which cells are seeded to grow. The growing cells closely mimic the natural native morphology and facilitate the development of intercellular networks and communication pathways.

- A variety of 3D polymer networks, including gel and sponge methods such as hydrogels, matrigel, and electrospun silk mats, offer convenient methods for 3D cultures of tissue-specific cells.
- Leighton was the first to demonstrate that both normal and malignant cells penetrate **cellulose sponge**. The used gel or sponge provides the matrix for morphogenesis and cell growth. The cells penetrate these gels and sponges while growing.
- One of the highly used 3D scaffold materials is **collagen gel**. This collagen gel provides the matrix for the culture of various mammalian cells such as epithelial cells. The morphogenesis of the cells and establishment of tissue-like morphology are possible using this type of scaffold. It was observed that the kidney-epithelial

cells (MDCK), if grown in collagen gel, respond to paracrine stimulation from fibroblasts via forming tubular structures.
- Since various 3D scaffolds are already discussed in the previous chapter (chapter ► "Mammalian Cell Culture in Three Dimensions: Basic Guidelines"), they are not discussed here.

8.1.3 Droplet/Cell Suspension Technique to Seed the Cells into a Three-Dimensional Scaffold

- Once the cells are isolated and a scaffold is ready, the cells are seeded using the **droplet technique** or **cell suspension technique**.
- The **droplet technique** involves pipetting a cell solution onto the scaffold at a **slow and constant rate**.
- In the **cell suspension technique**, the scaffolds are submerged in a cell suspension. The shaking of the scaffolds and the cell suspension may encourage cell migration into the matrix.
- The bioengineered constructs with high cell densities are the products of both techniques.

8.1.4 Incubation and Culture of Cells into Scaffold

- One can prepare a scaffold using any of the above materials. After the scaffold preparation, the cell seeding is optimally initiated.
- Take one 96-well mammalian cell culture plate.
- Aseptically transfer one sterile scaffold per well.
- Immediately add 400 μl cell culture medium to each well.
- Incubate in a CO_2 incubator for 30 min to equilibrate the spheroid with the medium.
- Aspirate the excess medium.
- Add the cell suspension to be cultured on the spheroids.
- Incubate the plate in a CO_2 incubator overnight to attach the cell to the scaffold.
- Next day, carefully remove the nonattached cells and 200 μl fresh culture medium per well.
- Allow the porous scaffold to be grown at a high cell density.

8.1.5 Use of the Cultured Scaffolds

- The purpose of the histotypic culture is to create a model system that closely resembles the in vivo physiological system and therefore cellular microenvironment. This 3D model system is utilized to study the behavior of the cells under various conditions. For example, to understand the process and mechanism of differentiation as well as regeneration of the stem cells, 3D scaffolds can be used. The stem cells grown in 3D scaffolds would give significant information about the various aspects of stem cell biology.
- The 3D scaffolds may be experimental subjects to various mechanical loads equivalent to that of natural tissues in the in vivo environment.

- The compression and biaxial loads applied during the cell growth may provide a preconditioned bioengineered tissue scaffold with a cellular structure resembling the native tissue.
- The major use of the scaffolds with engineered tissues is to replace or repair the damaged or defected tissues. The scaffolds (with engineered tissues with developed vasculature) are implanted into the defective region of the body and allow that region to grow.
- Gradually the scaffolds would be degraded and replaced by the newly formed natural matrix by the implanted, engineered tissues (Eberly et al. 2009).

8.1.6 Assets and Cautions of the Histotypic Culture

- Development of a cell line over several generations.
- Scale-up feasibility.
- Absolute control of the physical environment.
- Sample homogeneity.
- The low nutrient content is needed compared to that of animal models.

Cautions

- Cells may lose some differentiated characteristics.
- Cells are hard to maintain in the histotypic culture.
- Provides small tissue growth at a high cost.
- De-differentiation possibility.
- Instability/aneuploidy.

9 Organotypic Culture of Cells/Tissues

- In an organotypic culture, various cells having different lineages are cultured in a proper ratio and spatial relationship that resemble the in vivo conditions. This produces an organ component under laboratory conditions.
- So, the difference between organ and organotypic culture is that the former involves a part or section of the original organ for culturing in in vitro conditions without separating any cells from the organs. On the contrary, **organoid culture** involves culturing of the entire organ.
- Whereas in organotypic culture, the various cells isolated from the organs are cultured separately under the in vitro conditions before being mixed in a proper ratio and cultured over a 3D scaffold to grow and form an original organ-like structure.
- Additionally, the organ culture endows survival till 3 weeks as usual, but it does not allow the feasibility of further propagation.

So, for organotypic culture the following knowledge is highly essential:

1. Proper complex architecture of an organ.
2. Various constituent cells in an organ with their specific functions.

3. The ratio at which all the cells are present in an organ.
4. Type of common matrix preferred by all constituent cells of an organ.
5. Ability to culture the cells separately to get sufficient cell population in proportions having correct matrix or scaffolds.

- ***The basic purpose of the organotypic culture is to synthesize/construct a tissue/organ equivalent and to understand cell-to-cell crosstalk and cell-matrix crosstalk under both physiology and pathophysiology.***
- To simulate a tissue equivalent or multilayered cell culture, various methods can be used such as via **monolayer perfusion** (Kruse et al. 1970) to a complex **perfused membrane** (Klement et al. 1987) or **capillary beds** (Knazek et al. 1972).
- For organotypic culture, cells have to be separated/disaggregated and purified from organs, then mixed in a proper ratio to culture in 3D scaffolds.
- The best success in organotypic culture came from synthesizing the **skin equivalent** (Michel et al. 1999; Schaller et al. 2002; Regnier et al. 1997; Laning et al. 1999).

9.1 Organotypic Cultures in Epidermal Research

- Skin tissues are one of the most exposed tissues of the human body. For various reasons, human skin can damage and needs to be replaced, as in burn patients. Additionally, epidermal disorders including melanoma and psoriasis are among the most prevalent, often life-threatening, alongside having mechanistically complex pathologies.
- In its very basic form, the skin consists of a collagen-rich stroma dominated by fibroblasts and topped with a stratified epidermis. So, the major cells present in the skin are **fibroblasts impregnated into stratified epidermis** and **keratinocytes**. **Other cells like** melanocytes, Merkel cells, and Langerhans cells are also present.
- The key step in the skin reconstruction by the self-assembly approach is to use fibroblasts capable of secreting a mature ECM and keratinocytes being associated with one another to form a stratified, differentiated epidermis.
- Determining the most efficient way for extracting multiple cell types from a single cutaneous biopsy is to incubate the biopsy sample with different proteolytic enzymes at varying temperatures and time durations.
- Organotypic culture of skin cells can efficiently replicate the 3D region within which the dermatological cells can survive and function normally, proportionate to in vivo environment. The usual commencement of epidermal organotypic culture happens with the addition of fibroblast support cells to the vacant scaffolds. These scaffolds could vary from the freshly fabricated collagen gel in the

laboratory to the manifold commercial-grade matrices, and even the skin dermis priorly eliminated off its native cells. The transferred fibroblasts are provided an optimum time to enrich the scaffold, after which the epidermal keratinocytes are seeded over as the topmost layer. Henceforth, stratified differentiation of keratinocytes is triggered via upfronting the scaffold above the air-liquid interface (Oh 2013).

- As soon as a culture technique is activated, the behavior of the human epidermis can be analyzed and assessed under diversified diseased conditions. For example, *Ridky and colleagues* (2010) engineered the oncogene-powered epidermal neoplasia by replacing the normal epidermal cells with virally transduced cells (amid an organotypic culture) for overexpression of mutant cell cycle proteins.
- This created a way for a bypassing of normal cell checkpoint mechanisms by the transduced epidermal cells, resulting in mimicking of genetic manipulations frequently witnessed in the spontaneous in vivo malignant transformation of human epidermal cells. Such an in vitro epidermal neoplasia model made way for reproducing the basement membrane invasion, a prominent step in the in vivo metastasis of epidermal cancer cells.
- The rapidity and ease of understanding the establishment of an organotypic culture under the standard laboratory conditions propel them as the optimum system to study in the research projects aimed toward understanding the signaling mechanisms and monitoring the therapeutic efficacy of multiple drugs for human epidermal disorders.
- In their analysis, *Ridky and associates* (2010) also demonstrated the capability of epidermal neoplasia organotypic culture for a discrete validation of cancer inhibitors based on their potential to construct the basement membrane invasion. The same conditions were also found suitable to screen the anticancer potential of soluble peptides, neutralizing antibodies, or small hairpin RNAs.
- Several other cells (apart from fibroblasts and keratinocytes) could also be screened and evaluated concerning the skin organotypic culture. For instance, adding normal or malignant melanocytes can provide knowledge about the mechanisms of epidermal pigmentation or melanoma progression (Eves et al. 2000). Furthermore, the immune cells such as macrophages can be supplemented to the genotypically cultured scaffolds to analyze the more complex in vivo epidermal-dermal-immune signaling in the in vitro conditions (Bechetoille et al. 2011).
- Besides the skin, some other organs have also been modeled (grown) in organotypic cultures, such as **prostate and prostate cancer, breast and breast cancer, and lung and lung cancer**.
- Some important research attempts in the field of organotypic culture **include human esophageal epithelial cell 3D culture** (Kalabis et al. 2012), **tubular organotypic culture human kidney model** (Jun et al. 2018), and **organotypic brain slices culture** (Humpel 2015).

10 Organotypic Culture Techniques and Their Merging

To overcome the various methods used in organotypic culture, a combination of different methods and the development of "**organ on a chip**" have been made. In this method, the microfluidic technologies on a chip approaches deliver a constant steady-state culture medium flow to the tissues.

At present, reconstituted endothelial cells with microvascular development by bioengineering create tremendous hope to understand microvascular biology.

> As and when these networks are allowed to anatomize the pre-engineered microfluidic channels, this approach can support *in vivo* perfusion and physical scaling of OCS size, presently significantly limited owing to the passive diffusion inefficiency. (Sakaguchi et al. 2013)

10.1 Benefits of Organotypic Culture

- Human cells can be utilized for organotypic culture. Otherwise, the direct use of human subjects for experimental purposes is not only unsafe but also unethical.
- Organotypic culture helps to understand cell-to-cell crosstalk, cell-to matrix interaction, the complex behavior of various cells within a tissue, and cell signaling including bidirectional signaling (inside out and outside in the cells).
- It may be useful for understanding the developmental mechanism of various disease conditions.
- The efficacy and therapeutic potential of various experiments can be enhanced using the organotypic culture (Shamir and Ewald 2014).

10.2 Limitations of Organotypic Culture

- It is yet to develop a complete organ using organotypic culture. Therefore, the recapitulation of only a part of a normal organ may not reflect the overall structure and functions of an organ.
- The inefficient nutrient supply to the organotypic culture limits the size of an organotypic culture.
- In vitro study of diseased states requires analyzing tissues from disease-affected donors.
- In the present scenario, it is very challenging in terms of integration, analysis, modeling, and ultimately application of the data generated in such a complex three-dimensional culture model.
- It is essential to receive approval from specific patients to isolate induced pluripotent stem cells for the efficacy and testing of human-relevant drugs and other molecules (Esch 2015).

- At present, computational approaches are employed to reliably extrapolate the tissue model responses to the whole organism and provide testable predictions (Mortensen et al. 2016).

11 Benefits of Organ, the Histotypic and Organotypic Culture

The followings are the benefits of organ, histotypic, and organotypic cultures:

1. Organ culture provides a better and more accurate reflection of an organism's physiology.
2. Organ, histotypic, and organotypic cultures help to develop the behavioral interactions between cells within an organ, their growth characteristics in three-dimensional matrixes, or the process of organ development from simple cells. **The aim of these culturing configurations involves the creation of in vitro models comparable to the in vivo system.**
3. Organ culture is necessary because it is impossible to study various in vivo cellular interactions occurring in isolated cells or cultures.
4. Metabolism of the cells within an organ can also be studied using organ culture.
5. Organ cultures are also used to examine the effects of various **drugs, cytokines, chemokines, toxins, and other experimental agents** that limit the use of animal sacrifice.
6. This type of culture helps to understand the development of the human tissues and organs including **growth patterns, differentiation,** and **morphogenesis**. In this case, the various biochemical factors that influence proliferation, differentiation, and morphogenesis can be characterized.
7. The functions of these cultured tissues/organs under both physiology and pathophysiology can be examined.
8. It may give some clues about the developmental anomalies of various organs.
9. These cultures considerably reduce the number of experiments necessary for culturing whole animals to investigate a given problem.
10. Scientists from various **Regenerative Medicine Institutes anticipate the growth of the complete structure of various organs,** useful for human transplantation.

The most important organs on the verge of experimental development are as follows:

A jaw bone by Columbia University.
A lung by Yale University.
Beating rat heart by Minnesota University.
A kidney by Michigan University.
Heart perfusion w/stem cells created a "new heart" by the University of Minnesota in 2007.

12 Limitations of Organ, Histotypic, and Organotypic Culture

The success rate of developing a complete organ by organ culture is very much limited due to the following reasons:

1. The major and most important limitation of organ culture is the lack of proper vasculature in the growing organ. Since nutrients and oxygen do not diffuse properly to the core or center of the organ, these cells eventually die via necrosis.
2. Another major limitation of organ culture is its nonpropagated property. Every time experimenting, one needs to start with a fresh sample; therefore, reproducibility of the data is difficult and expenditure cost is high.
3. Finally, experimental results of in vitro cultured organs with drugs, toxins, and other agents are often not comparable to those from in vivo studies (e.g., studies on drug action) since the drugs are metabolized in vivo.
4. Despite all these limitations, organ culture is one of the most important research domains, notably for **basic developmental biology, regenerative medicine, transplantation immunology, and pharmaceutical biochemistry**.
5. Newer culture approaches, such as **3D bioprinting, organoids, or organs-on-a-chip** attempt to improve the replication of tissue microenvironment, although limited success at present promises a big hope for the future.

13 The Concept of Tissue Engineering and Regenerative Medicine

- A commonly applied definition of tissue engineering as stated by ***Langer*** *and* ***Vacanti*** is as follows:

  "An interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes capable of restoring, maintaining or improving functions of biological tissues or an organ, as a whole."
- Tissue engineering is an in vitro process that produces functional tissue (closely resembling the natural tissue) by seeding cells into a biomaterial matrix, called **scaffolds** before subsequent implanting of generated tissue in the body.
- For incorporation into scaffolds, remodeling is necessary but stress-induced architecture cannot be remodeled. In this process, since the tissue is generated in vitro, there is every possibility of checking its viability under the in vitro conditions. As a multidisciplinary field, tissue engineering combines **biology, biochemistry, clinical medicine, and materials science** to achieve clinically targeted results (Fig. 7).
- In contrast, **regenerative technology** generally denotes implantation of the biomaterial matrix with or without seeded cells into scaffolds, directly into the body to facilitate in vivo regeneration of the damaged/injured/diseased tissue.
- In this process, the incorporation and formation of tissues are under the influence of endogenous regulators (including mechanical strain).

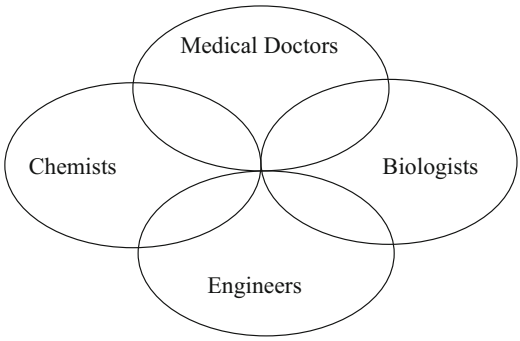

Fig. 7 Multidisciplinary essence of tissue engineering

- Additionally, in this process, since the cells are directly seeded into the body, there are no chances of a prior experiment regarding the feasible or infeasible tissue effectiveness under the in vivo conditions.
- Moreover, enough chances are there that the implanted tissue may be dislodged or degraded by mechanical stresses under the in vivo conditions.
- For repairing the damaged tissues, the complications associated with the conventional organ donation procedure can be compensated by tissue engineering and regenerative medicine.
- This technology is used to create cells that aid in the healing of diseased or damaged organs.
- At present, tissue engineering becomes an alternative in clinical medicine.
- While at present, through tissue engineering it is not possible to generate a complete organ, the ultimate target is to synthesize various organs completely that could be utilized to better the survivability chances (Langer and Vacanty 1993; Meyer et al. 2009; Lanza et al. 2011; Li et al. 2016).

The section on tissue engineering has been discussed in the following parts:

1. The usefulness of tissue engineering and its applications
2. History of tissue engineering
3. Elements of tissue engineering
4. The process of tissue engineering
5. Current status of tissue engineering

The above points are discussed in the subsequent paragraphs:

13.1 The Usefulness of Tissue Engineering and Its Applications

In today's world, tissue engineering is a necessity because of the following reasons:

- In today's world, there is a large demand for the supply of donor organs. However, the supply of donated organs seldom matches that of the requirement. Under this condition, tissue engineering may play a very important role in the supply of engineered organs.
- Since other available therapies such as synthetic prostheses, drug therapy, surgical reconstruction, and medical devices are not always successful, tissue engineering may be very useful.
- Congenital abnormalities require tissue engineering.
- Most tissues cannot regenerate following a disease or injury.
- Even tissues that regenerate spontaneously may not be entirely efficient.
- Permanent implants have a lot of success but also carry numerous problems.
- Tissue engineering holds promise for the generation of better transplantable organs.
- It is claimed that, shortly, tissue engineering may be useful against various genetic defects.
- The major goal of tissue engineering is the in vitro construction of various tissues and organ parts.

13.1.1 Tissue Engineering May Have the Following Additional Applications

- Tissue engineering has diagnostic applications and is utilized as a biosensor.
- At present, tissue engineering is used to repair or replace tissues such as **urinary bladder, heart valve, blood vessels, bone, cartilage,** etc.).
- The subjects of tissue engineering and **regenerative medicine** are closely interlinked and aim collectively at the development of functional cells, tissues, and organ substitutes to repair, replace, or enhance the lost biological function (s) to **congenital abnormalities, injury, disease, or aging**. Thus, the regeneration of tissues is one of the most important applications of tissue engineering.
- The engineered tissues can be used as a **biosensor**.
- Drug development is a complex process, comprising screening of novel molecules, identification of genes as potential targets, evaluation of the drug metabolism, uptake, and possible toxicity risk.
- Tissue engineering employs living cells as potential engineering entities. For example, artificial skin that comprises viable fibroblasts, cartilage repaired with living chondrocytes, or several other cells that could be engineered via specific routes.
- A befitting case pertains to engineered heart valves made using tissue engineering for an amicable replacement of their nonfunctional states. This is mediated by bypassing the limitations confronted with currently used bioprosthetic and mechanical heart valves. Tissue-engineered skin enables noted improvements in wound healing, taking care of the limitations of autograft usage.

The general usefulness of tissue engineering is as follows:

- Helps a person conquer a disease or illness.
- Fewer surgeries are needed by the person being treated.

- No or fewer rejection chances.
- Organ failure sufferers do not have to wait for an organ donor.
- The need for organ donation after death could be eliminated.
- This technology could lead to even greater and better future technologies.
- The technique provides a permanent solution for tissue problems.

13.1.2 Major Drawbacks and Hurdles of Tissue Engineering

- A lot of research work is needed to contract a tissue because it is very difficult to construct tissues. Cells have to stay alive inside the body and should continue to function, a criterion that is optimized with significant difficulty for complex organs.

Some limitations of successful tissue engineering are as follows:

- Size of defect, e.g., the bone does not regenerate in large defects.
- The collapse of surrounding tissues to the defect is often visualized. For example, periodontal defects.
- Excessive strains in the reparative tissue are often noticed. For instance, the unstable nature of fractures (Trowell 1961).

13.2 History of Tissue Engineering

- In 1970, the early experiments of tissue engineering were demonstrated by ***W. T. Green*** **amid an attempt** to generate cartilage. For his experiment, Dr. Green used chondrocytes and seeded these cells onto spicules of bone before finally implanting them into nude mice. Although unsuccessful, Dr. Green's experiment paved the way to generate new biomaterials for seeding the cells in tissue engineering.
- In the 1980s, doctors ***Burke*** and ***Yannas*** of the Massachusetts General Hospital and M.I.T. used collagen matrix for the growth of human dermal fibroblasts both in a laboratory experiment and in human model studies.
- Dr. ***Howard Green*** later transferred keratinocyte sheets onto burn patients.
- Additionally, Dr. ***Eugene Bell*** seeded collagen gels with fibroblasts, referring to them as contracted collagen gels.
- All these examples represent seeds of the new discipline, now known as "Tissue Engineering."
- In 1984, ***Wolter*** *and* ***Meyer*** **first used** the term tissue engineering while working on an endothelium-like layer on PMMA in the eye.
- Additionally, in 1988 Prof. ***Robert Nerem*** at a symposium at the University of California Los Angeles (UCLA) used the term tissue engineering for the first time.
- In the mid-1980, the research works of Dr. ***Joseph Vacanti*** and Dr. ***Robert Langer*** significantly progressed the field of tissue engineering by publishing some important papers.
- At this juncture, the major domains in which *Dr. Vacanty* worked include the design and implementation of functional tissue equivalents. For this purpose, he used synthetic biocompatible/biodegradable polymer comprising branching

network, configured as scaffolds seeded with viable cells. Interestingly, in 1988 at a meeting of the American College of Surgeons, *Vacanti and colleagues* published significant findings in the area of tissue engineering, which was published in Surgery.

- In 1991, ***Cima, Vacanti,*** and ***Langer*** cultured chondrocytes on a PGA scaffold, in the ears of the nude mouse.
- Henceforth, in 1993, the publication of *Vacanti and associates* in Science provided comprehensive knowledge about tissue engineering.
- In the field of tissue engineering, several specialized centers developed in the USA and Europe. In the early 1990s, ***Peter Johnson*** established Pittsburgh Tissue Engineering Initiative (**PTEI**).
- Similarly, ***Robert Nerem*** at the Georgia Tech laboratories developed **cardiovascular tissue engineering**.
- Erstwhile in the USA, the other tissue-engineering laboratories were developed at Rice University in Houston and the UMass Medical School.
- In the UK, Dr. ***Julia Polak*** at the Imperial College of London organized a British-origin society that formed a loose association with the Tissue Engineering Society (**TES**), being previously incorporated in Boston.
- In the same 1990 period at Giesen, Germany, Dr. ***Una Chen*** started tissue engineering and stem cell research.
- Dr. ***Clemente Ibarra*** at the National Institute for Rehabilitative Medicine in Mexico formed the Mexican Tissue Engineering Society and founded laboratories for tissue engineering.
- In 1994, ***Brittberg*** *and* ***Peterson*** published a paper in the New England Journal of Medicine (NEJM) on the implantation of human autologous chondrocytes.
- A tissue-engineering laboratory was established by Dr. Wolfgang Pulacher in Innsbruck at the Leopold Institute. At the same time, the establishment of laboratories by the combined efforts of Germany, Switzerland, and Southern France (Dr. Raymund E. Horch and Dr. G. B. Stark) gained swift momentum at the University of Freiburg. The combined efforts resulted in the formation of the German Tissue Engineering Society in Western Europe.
- It was by the late 1990s, that Dr. R. Hetzer (a cardiovascular surgeon at the University of Berlin) and Dr. Christof Brelsch (a liver transplant specialist in Hamburg) collaborated with the Children's Hospital in Berlin as also with Dr. Koichi Tanaka's group from the University of Kyoto.
- As far as Asian participation is concerned, Dr. Minora Ueda of Nagoya University, Japan, set up a large Tissue Engineering facility. This happened during the first meeting of the Japanese Tissue Engineering Society (1997) in Nagoya.
- Inceptive efforts from China for Tissue Engineering were also encouraged by their Governmental grant, founded by *Dr. Yi Lin Cao* in Shanghai (Vacanti 2006; Vacanti and Vacanti 2007; Berthiaume et al. 2011).

13.3 Elements of Tissue Engineering

Mammalian tissues are composed of the following materials:

13.3.1 Cells for Tissue Engineering

Implanted and cultured cells are capable of creating new tissues.

13.3.2 Insoluble Extracellular Matrix

Various biomaterials act as a scaffold or matrix, holding the cells and supporting growth.

13.3.3 Soluble Molecules That Serve as Regulators of Cell Functions

Biological signaling molecules instruct cells to form desired tissue types (Fig. 8).

Ahead is the brief discussion of the above three components:

Cells for Tissue Engineering

Cells are the major components of tissue engineering. Cells may be originated from various sources (Naderi et al. 2011; Caplan 2007).

Based on their sources, cells are divided into the following types:

A. Autologous cells
B. Allogeneic cells
C. Xenogenic cells
D. Isogenic/syngeneic cells
E. Primary cells
F. Secondary cells

Here is a brief discussion of the above cell types:

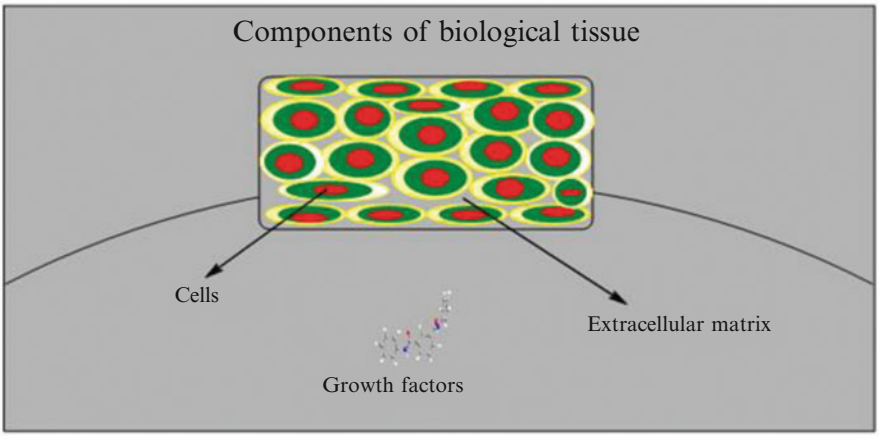

Fig. 8 A workable tissue-engineering model requires optimum contribution from all the depicted three factors (cells, growth factors, and ECM)

Autologous Cells

These cells are obtained from the same individual to which they are reimplanted. In the use of these cells, there is no issue of rejection. The availability of cells is a major concern here.

The main advantages of autologous cells in tissue engineering are as follows:

- Avoiding immune complications
- Reduction in the possible transfer of inherent infections

There are certain disadvantages associated with autologous cells:

- It is not always possible to obtain sufficient biopsy material from the patient.
- Diseased state and patient age are the often encountered limiting factors.

Allogeneic Cells

These cells originate from a donor body within the same species. This infers that if the cells are taken from a person other than the patient, the source is often allogeneic. While there are some ethical constraints about in vitro utility of human cells, the employment of dermal fibroblasts from the human foreskin has been demonstrated as immunologically safer (Wystrychowski et al. 2014).

The main advantages of allogenic cell source are as follows:

- Obtained in good quantity from a healthy donor.
- Can be cultured on a large scale.
- Cost-effective with consistent quality.
- Available, as and when required by a patient.

The **major problem** of allogeneic cell sources pertains to immunological complications that may ultimately lead to graft rejection. The immune responses, however, are variable depending on the used cells. For instance, endothelial cells are more immunogenic than fibroblasts and smooth muscle cells. The age of the donor is another important factor that contributes to immunological complications. The cells from adult donors are highly immunogenic, while fetal or neonatal cells elicit little or no immune response.

Xenogenic Cells

These cells are isolated from individuals of another species. In particular, animal cells are quite extensively used in experiments aimed at the design of cardiovascular implants.

Isogenic/Syngeneic Cells

These cells are isolated from genetically identical organisms, such as twins, clones, or highly inbreed research animal species. Cells from immunologically compromised syngeneic mice could also be used experimentally.

Primary Cells
Any kind of cells originated from whatever source on being cultured for the first time.

Secondary Cells
These cells come from a cell bank or subsequent culture of the primary cultured cells.

***Based on potency, the cells are divided into the following types*:**

Stem cells
Totipotent cells
Pluripotent cells
Multipotent cells
Oligopotent cells
Unipotent cells

Here is a brief discussion of the above cell types:

Stem Cells
These cells are undifferentiated with an ability to divide in culture and give rise to different specialized cells. According to their source, stem cells are divided into the following types.

Totipotent Cells
These cells can proliferate and differentiate into a complete body. A **totipotent cell** can give rise to all extraembryonic tissues, along with all body tissues including those of the germline.

Pluripotent Cells
Pluripotent stem cells are cells having the self-renewal capacity by dividing and developing into the three primary germ cell layers of the early embryo and therefore into all cells of the adult body, but not extraembryonic tissues, like the placenta.

Multipotent Cells
Multipotent stem cells are cells possessing the self-renewal capacity by dividing and developing into multiple specialized cells present in a specific tissue or organ. Most adult stem cells are multipotent stem cells.

Oligopotent Cells
The oligopotent cells are the progenitor cells that have the limited ability or potency to differentiate into only a few kinds of cells, for example, lymphoid or myeloid stem cells.

Unipotent Cells
"**The word** '**uni**,' meaning **itself**, is derived from the Latin word '**unus**,' meaning **one**." Thus, this kind of progenitor cell has a very limited capacity to differentiate into only one cell type, i.e., lowest differentiation potential.

13.3.4 Insoluble Extracellular Matrix

- The extracellular components of **ECM** support the attachments or adhesions and stabilize the cells in tissue architecture, thereby helping in their growth.
- An ECM consists of various proteins including **collagen, laminin, fibronectin glycoprotein, and proteoglycan**.
- The prominent receptor molecules of the intracellular membrane receptors that interact with the ECM and send bidirectional signals between cells and ECM are **integrins**. **These** are the proteins that function mechanically, by attaching with cell cytoskeleton to the ECM and biochemically, by sensing the adhesion. The **integrin** family of proteins consists of α and β subtypes, collectively forming transmembrane heterodimers (Table 1) (Williams 2019).

13.3.5 General Characteristics of the Extracellular Matrix

The ECM has the following general characteristics:

- In the ECM, various molecules (**including cell-adhesive/cell-signaling molecules**) are interconnected to form complex large structures and therefore possess low diffusion ability.
- The ECM molecules are capable of bidirectional signaling (**inside out and outside in**) across the cells.
- These molecules influence the various physiological events of the cells that include differentiation, apoptosis, migration, etc.

Table 1 Here is the profiling of various integrins present in the cell membrane

Integrin	Ligands
$\alpha_1\beta_1$	Collagen (I, IV, and VI), laminin
$\alpha_2\beta_1$	Collagen (I–IV, VI), laminin
$\alpha_3\beta_1$	Collagen (I), laminin, fibronectin, entactin, and epiligrin
$\alpha_4\beta_1$	$\text{Fibronectin}_{\text{ALT}}$, VCAM-1, and thrombospondin
$\alpha_5\beta_1$	Fibronectin, thrombospondin
$\alpha_6\beta_1$	Laminin
$\alpha_7\beta_1$	Fibronectin
$\alpha_L\beta_2$	ICAM-1, ICAM-2, and ICAM-3
$\alpha_M\beta_2$	ICAM-1, iC3b, fibrinogen, factor X, and denatured protein
$\alpha_V\beta_3$	Fibrinogen, iC3b, and denatured protein
$\alpha_V\beta_1$	Vitronectin, fibrinogen, fibronectin, and thrombospondin
$\alpha_V\beta_3$	Vitronectin
$\alpha_V\beta_5$	Fibrinogen
$\alpha_V\beta_6$	Laminin
$\alpha_0\beta_4$	$\text{Fibronectin}_{\text{ALT}}$, VCAM-1, and MAdCAM-1
$\alpha_4\beta_7$	Fibrinogen, fibronectin, vitronectin, and vWF
$\alpha_1\beta_3$	Fibrinogen, fibronectin, vitronectin, vWF, collagen (IV), and entactin

vWF von Willebrand factor, *ICAM* intracellular cell adhesion molecule, *VCAM-1* vascular cell adhesion molecule, *MadCAM-1* mucosal addressin cell adhesion molecule I

13.3.6 Scaffolds

- Biochemically, the scaffolds comprise either natural biomaterials such as collagen or synthetic materials such as polyglycolic acid. Both these materials are porous and absorbable.
- Technically, scaffolds are artificial structures suitable to support the three-dimensional culture and mammalian cell growth.
- ***Scaffolds are used to guide, organize, grow, and differentiate the cells amid the formation of functional tissue. Scaffolds also facilitate the delivery of both physical and chemical signals to the growing cells*** (Sultana 2003).
- The details about the scaffolds are already discussed in the previous chapter (chapter ▶ "Mammalian Cell Culture in Three Dimensions: Basic Guidelines").

13.3.7 Soluble Molecules That Serve as Regulators of Cell Functions

- Signaling molecules that help in the growth of cells in a scaffold are divided into three distinct groups, as ahead.
- Growth and differentiation factors such as PDGF, IGF, TGBF, fibroblasts growth factors (FGFs), Bone Morphogenetic Factors (BMFs), etc.
- ECM proteins and attachment factors.
- Mediators of bone metabolism.

13.4 The Process of Tissue Engineering

The purpose of tissue engineering is to generate a 3D construct that is structurally, mechanically, and functionally similar or even may be better than its normal/natural tissue counterparts. The process involves the creation of natural/synthetic cell culture support or scaffolds with a biodegradable essence to grow mammalian cells in a 3D configuration.

The process of tissue engineering involves the following steps:

A. Culture of isolated cells.
B. Preparation of scaffolds from various biomaterials: scaffold fabrication.
C. Seeding of scaffold with living cells.
D. Bathe scaffold with growth factors.
E. Cell multiplication, scaffold filling, and growth into three-dimensional tissue.
F. Implant the generated tissue inside the body.
G. The intended natural functions of tissue would be recreated by the constituent cells.
H. The tissues will be attached and connected with the new blood vessels.
I. Gradually and slowly, the scaffolds would be degraded.
J. Eventually, the newly grown tissue blends with the surrounding tissues.

- Very briefly, specific mammalian cells are isolated and cultured. Simultaneously, scaffolds are synthesized. Thereafter, the scaffolds are seeded with the desired cells before being bathed with nutrients, growth factors, etc. for adequate cell

growth. Cells are, thereafter, allowed to grow in scaffolds, simultaneously acting as 3D tissues. Finally, the generated tissue would be implanted into the mammalian body.

- The methods adopted for culturing the cells in tissue engineering depend on the cell type and functions. For most cells, the conventional monolayer cultures either in Petri dishes or T flasks serve the purpose.
- The major drawback of monolayer cultures is that the cells lose their morphology, functions, and proliferative capacity after several generations.
- Some workers prefer 3D cultures for the cells in tissue engineering using large-scale culture containers such as bioreactors.
- The nutrient and gaseous exchanges may be limiting factors in 3D cultures. Therefore, proper standardization of the cell culture medium is necessary.

13.4.1 Cell Orientation

The orientation of cells about specific shape and spatial arrangement is influenced by the following environmental factors:

- Substrate or contact guidance
- Chemical gradients
- Mechanical cues

13.4.2 Substrate or Contact Guidance

The topographical features of the substrate determine the contact guidance. These features may be in the form of ridges, aligned fibers, etc. It is possible to use differential attachment mode to substrates, as the means of producing different cell alignments. In recent years, synthetic polymer substrate, collagen fibrils, and fibronectin have been used as bioresorbable templates for tissue engineering.

13.4.3 Chemical Gradients

The development of chemical gradients is required for cellular orientation and the stimulation of cellular functions. Certain growth factors and extracellular macromolecules are capable of creating chemical gradients, e.g., vascular endothelial growth factor (VEGF), oligosaccharide fragments of hyaluronan, fibronectin, and collagen. There are certain practical difficulties in maintaining the optimum chemical gradients for the cells in 3D cultures. This is particularly the limiting factor when the cells become dense.

13.4.4 Mechanical Cues

The response of cells to mechanical signals is complex and may result in any one or more of the following:

- Changes in the cell alignment.
- Deformation of the cytoskeleton.
- Altered matrix formation.
- Synthesis of regulatory molecules (e.g., growth factors, hormones).

There are mainly three mechanical cues governing cell populations

- Tensional forces
- Compression forces
- Shear forces

13.4.5 Design and Engineering of Tissues

The following surgical criteria are taken into consideration while dealing with tissue engineering:

- Rapid restoration of the desired function
- Ease of fixing the tissue
- Minimal patient discomfort

For the proper conduct of tissue engineering, the source of donor cells plays a decisive role. The use of patients' cells (autologous cells) eliminates multiple immunological complications. Allogeneic cells are also used, particularly when the tissue engineering construct is designed for a temporary repair. It is observed that when the cells are cultured and/or preserved (i.e., cryopreservation), the antigenicity of allogeneic cells is reduced. Another important criterion in tissue engineering is the support material, its degradation products, cell adhesion characteristics, and mechanical cues (Caddeo et al. 2017).

The design and tissue engineering concerning skin, urothelial, and peripheral nerve are briefly described hereunder.

13.4.6 Tissue-Engineered Organ Types

Tissue-Engineered Skin

It was first demonstrated in 1975 that human keratinocytes could be grown in the laboratory in a form suitable for grafting. Many improvements have since been made, enabling the growth of epithelial cells and producing a continuous sheet that progressed to form carnified layers.

The major difficulty encountered while tissue engineering skin is the isolation of the dermal layer possessing blood capillaries, nerves, sweat glands, and other accessory organs. The recent past has witnessed some developments for generating implantable skin substitutes, regarded as tissue-engineered skin constructs (Bell et al. 1981).

Integra

This is a bioartificial material composed of collagen-glycosaminoglycan. Integra is not a true tissue-engineering construct. It is mainly used to carry the seeded cells.

Dermograft

This is composed of poly (glycolic acid) polymer mesh seeded with human dermal fibroblasts derived from neonatal foreskins.

Apigraft

This has human dermal fibroblasts seeded into the collagen gel. A layer of human keratinocytes is then placed on the upper surface. The tissue constructs described above have a limited shelf life (about 5 days). However, they can integrate into the surrounding normal tissue and form a good skin cover. To date, there is no evidence of immunological complications with tissue-engineering constructs.

13.4.7 Tissue-Engineered Urothelium

It is now possible to culture urothelial cells and bladder smooth muscle cells. This raises the hope for the feasibility of tissue-engineering urothelium construction. Considerable success has been reported in the development of a functional bladder in dogs.

For this purpose, a poly (glycolic acid) polymer base was shaped into a bladder with an outer surface coating of muscle cells. The luminal surface (i.e., inner surface) is coated with precultured urothelial cells. The bladder constructed in this way functioned almost like a normal one and worked successfully even after being maintained for a year.

13.4.8 Tissue-Engineered Peripheral Nerve Implants

Peripheral nerve injury is a common hallmark of trauma and tumor resection surgery, lending significant feasibility to irreversible muscle atrophy. Therefore, the repair of injured peripheral nerves assumes significance.

A diagrammatic representation of the basic design of a peripheral nerve implant is depicted in Fig. 9.

The regeneration of the injured nerve occurs from the proximal stump to rejoin at the distal stump. The regeneration is guided by three distinct substances.

Conduct Materials

This is the outer layer and is the primary guidance source. Conduct material is composed of collagen-glycosaminoglycan, PLGA (poly lactic-co-glycolic acid), hyaluronan, and fibronectin. All these moieties are bioresorbable.

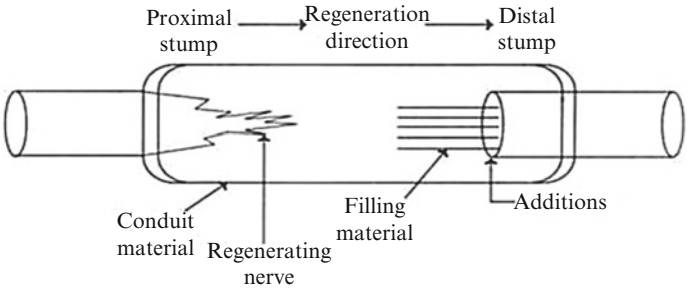

Fig. 9 Working configuration of a peripheral nerve implant

Filling Materials

This supports the neural cells for regeneration, besides guiding the regeneration process. Filling material comprises collagen, fibrin, fibronectin, and agarose.

Additives

Additives include a large number of growth factors, neurotrophic factors (in different forms, varying combinations), e.g., fibroblast growth factor (FGF), and nerve growth factor (NGF). Additions of Schwann cells or transfected fibroblasts promote the nerve generation process.

Tissue Modeling

Research is in progress to create tissue models as artificial organs. Some recent developments in experimental tissue modeling are briefly outlined.

Artificial Liver

Hepatocytes, cultured as spheroids or hepatocytes, and fibroblasts, cultured as heterospheroids, can be used. They are held in artificial support systems such as porous gelatin sponges, agarose, or collagen. The addition of exogenous molecules is useful for the long-term liver cell culture. Considerable progress has been reported in creating artificial liver as evident from the three-dimensional hepatocyte structure and metabolic functions.

Artificial Pancreas

Spheroids of insulin-secreting cells are being developed from mouse insulinoma beta cells. Some investigators implanted fetal islet-like cell clusters under the mice's kidneys, although the functions were not encouraging due to limited oxygen supply.

Pituitary Gland

Multicellular spheroids were created to study certain hormonal releases, e.g., luteinizing hormone (LH), following stimulation by luteinizing hormone-releasing hormone (LHRH). Some success has also been achieved to create spheroids for melatonin generation.

Thyroid Gland

Thyroid cell spheroids can be used for studying cell adhesion, motility, and thyroid follicle biogenesis.

Brain Cell Culture

Three-dimensional brain cell cultures are in use for the study of neural myelination and demyelination, neuronal regeneration, and lead neurotoxicity. Aggregated brain cells are also used for the study of Alzheimer's and Parkinson's diseases.

Heart Cell Culture

Aggregated heart cells are in use for the study of cardiac development and physiology. Important research publications in the development of various mammalian

organs, through the process of tissue engineering, include the mammalian **urinary bladder** (Oberpenning et al. 1999), **vagina** (De Filippo et al. 2003), **intestinal tissues** (Chen and Beierle 2004), **airway** (Macchiarini et al. 2008), **bone** (Porter et al. 2009), **musculoskeletal** (Nie et al. 2012), **myocardial tissue** (Vunjak Novakovic et al. 2014), **liver** (Lin et al. 2015), and many more tissues and organs.

13.4.9 Current Status of Tissue Engineering

Currently, the success rate of tissue engineering is limited.

The following limitations of tissue engineering are presently observed:

- Full regeneration of tissues that do not regenerate spontaneously has not yet been achieved.
- Lots of successes in bone tissue engineering.
- Tissue-engineered skin has no glands and hair.
- Engineered cartilage is not articulate.

13.4.10 Current Technology Review

The success of some of the companies in the tissue-engineering section is discussed in the following paragraphs:

The Mattek Corporation

- In 1985, two scientists from the Massachusetts Institute of Technology (MIT), USA, founded the **MatTek Corporation**, a tissue-engineering company.
- This company produced a human tissue model of the skin, ocular and respiratory systems.
- Soon, these tissue systems became popular throughout the pharmaceutical and cosmetic industries.
- Until recently, MatTek Corporation produces more than 13 products of which the most successful is the **EpiDerm system, an in vitro cell-based skin tissue system**.
- Now 80% of skin transplantation-based products employ EpiDerm.

The Hurel Corporation

Hurel Corporation, North Brunswick, is a New Jersey, USA-based company. The most promising product of Hurel is its hepatic cell coculture which is already in use by Merck company. These engineered cells are specifically produced for rigorous industrial use relying on the cells' manufacturability, ruggedness, convenience, and replicability. This coculture model is now useful for industrial testing of drugs and other molecules, their metabolism along with toxic effects on the hepatic cells. In addition, hepatic cocultures are utilized in the areas such as time-based dynamics of metabolites, clearance rates of compounds, and timed, repeat dosing for monitoring acute and chronic toxicities. Hopefully, with the advancement of this coculture technology, animal sacrifice for drug testing would be significantly reduced.

The Modern Meadow

This company was founded in 2011. This company proposed a new approach "to culture meat based on bio-printing, a computer-controlled assembly method for making meat tissue" (Mauney et al. 2005; Sharma et al. 2019).

14 Conclusion

Organ, the histotypic and organotypic cultures, and tissue engineering principles and techniques are briefly described in this chapter. Collectively, the basic purpose of these in vitro techniques is to produce various mammalian tissues or part of an organ or a complete organ, comparable to in vivo organs of the mammalian body. These tissues or organs could be used to replace old, damaged or injured, and diseased mammalian organs. While tremendous progress has been achieved in tissue engineering and the development of various organs using 3D scaffold procedures, there is still a long way to go for developing a complete mammalian organ. Intensive efforts in the field, 1 day, could enable the development of various mammalian organs, offering immense help to mankind and society.

15 Cross-References

► Isolation and Primary Culture of Various Mammalian Cells
► Mammalian Cell Culture in Three Dimensions: Basic Guidelines

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Large-Scale Culture of Mammalian Cells for Various Industrial Purposes

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_15-2

Abstract

There is the widespread use of large-scale mammalian cell culture in biotechnological and pharmaceutical industries for various purposes. Select vital provisions used to culture mammalian cells from moderately high to large scale are **Spinner Flasks, Cell Factories, Roller Bottle Systems, Hollow Fiber Systems, and Bioreactors**. The volume of the cell culture can be increased by increasing the number of Spinner Flasks, Cell Factories, Roller Bottle Systems, and Hollow Fiber Systems. However, mammalian cell culture bioreactors are available in various sizes, ranging from (1 to 5) liters (tabletop), (10 to 100) liters in medium sizes, or even to an extent of thousand liters. Prominent bioreactors, presently in use, include **Stirred Tank Bioreactors, Bubble Column Bioreactors, Airlift Bioreactors, Tower Bioreactors, and Fluidized Bed Bioreactors**. These large-scale instruments control and monitor various parameters such as effective nutrient supply, regular harvesting of products, and discard of toxic metabolites such as ammonia, aeration without generating air bubbles, creating shear stress, monitoring, and maintenance of optimal temperature (37 °C), pH (~7.4), moisture, pressure, and osmolarity within the culture medium. At present, very large-scale bioreactors are robotically controlled. Regarding culture procedures, suspension cultures are preferred over their adhesion counterparts due to high-density cell growth and increased generation of products. The different operational modes of a bioreactor in use for product harvest include batch and fed-batch continuous culture. Many research groups have developed novel bioreactors for growing specialized tissues/cells on a structural scaffold, in an attempt to recreate organ-like tissue structures in vitro.

Keywords

Mammalian cell culture · Bioreactors · Scale-up operation · Large scale culture · Adherent vs suspension culture · Batch and fed-batch (continuous culture)

1 Introduction

This chapter describes the large-scale mammalian cell culture for the production of various biological products. The primary objective of mammalian cell culture scale-up is to obtain a high yield of cells and the products thereof, from as small a cell-culture medium volume as possible. This mode of working reduces the cost of cell culture-based products, in turn benefitting the biotechnological, pharmaceutical, and biomedical/clinical consumers. In comparison to microorganisms, mammalian cells are large 10–30 μM, fragile (having only a plasma membrane and no cell wall) along with being highly sensitive to shear stress, accumulated metabolite concentration (such as **lactic acid**), and growth pace (~ 10–50 h **doubling time**). So, maintaining normal physiological conditions in a cell culture medium such as temperature (37 °C), moisture (95%), pH (7.4), O_2/CO_2 tension, reduced shear stress, elimination of toxic metabolic byproducts such as lactic acid, and removal of dead cells, is highly

essential. Additionally, **maintaining sterility all through the mammalian cell culture duration is essential**.

The major large-scale culture systems described in this chapter are **cell factories, roller bottle systems, hollow fiber systems, and bioreactors**. Each system has its specific advantages (merits) and disadvantages (demerits). Of the various large-scale systems, the use of the bioreactors is specially focused on in this chapter because of its widespread suitability from the industrial and clinical viewpoints. A bioreactor may refer to any device or system that supports a biologically active-controlled environment for culturing a large cell population using a large volume of culture medium. In general, a bioreactor can be termed a **fermenter** if it is optimized to produce acids, alcohol (ethanol), or other cell-based products via **fermentation**, through cultured bacteria/yeast/fungus or other **microorganisms**. **Though** broadly a bioreactor and a fermenter are the same, technically, some structural distinctions do prevail between a mammalian cell culture bioreactor and a fermenter. A mammalian cell culture bioreactor is characterized by a **marine impeller**, **round bottom, an aspect ratio (AR) of 2:1**, and a **water-jacketed temperature control system**.

Besides the main body, other basic structural components of a bioreactor include an **agitator**, **baffles**, **sparger**, and **jacket**. Several bioreactor configurations, namely, **stirred tank bioreactors, bubble column bioreactors, airlift bioreactors, fluidized bed bioreactors, packed bed bioreactors**, and **photo-bioreactors**, are presently available. Since there are so many bioreactors available to culture mammalian cells, sometimes it is difficult to select a particular model for a specific purpose. Small bioreactors are preferred for suspension culture while fixed bed tabletop bioreactors and fluidized bed reactors are mostly used for research purposes or process development. For larger product yields, only bioreactors equipped for suspension culture or, up to a certain scale, fixed or fluidized bed bioreactors have the requisite scalability. For large-scale production of various cell-based products, stirred tank bioreactors with suspension culture are mostly used because of less production cost.

Setting up large-scale culture process optimization requires consideration of the **cell type, nutrient medium, feeds, and bioreactor settings along with minimizing the by-product generation**. Two operational modes (**batch/fed-batch culture versus continuous flow culture**), as well as support systems (suspension culture versus adherent culture), are discussed in this chapter. Finally, basic steps involved in large-scale mammalian cell culture are presented (Arathoon and Birch 1986; Hu and Aunins 1997; Bliem and Katinger 1988a, b; Broad et al. 1989; Ramasubramanyan and Venkatasubramanian 1990). Overall this chapter will enhance the reader's knowledge about the large-scale mammalian cell culture.

2 Differences Between Small, Medium, and Large-Scale Mammalian Cell Cultures

- Mammalian cells can be cultured on small, medium, or large scales.
- For **small-scale** culture, **T flasks** are important.
- The **"T"** refers to the **total surface area of the flask** available for cell growth.

- T flasks are available in various sizes like **T-25, T-75, T-150, T-175, T-225, T-500**, etc. T-25 flask means it has a 25 cm^2 growth area.
- All the above flasks are made up of **polystyrene** and exhibit suitability for mammalian cell culture. The polystyrene-made culture plates of various sizes are also used for small-scale mammalian cell culture.
- Of note, mammalian cells' surface overall maintains a slightly negative charge, to which adherent mammalian cells attach well in the polystyrene-coated culture vessels.
- The perception of large scale may vary from person to person to person. For example, in some laboratory transfers of cell culture from a T-25 or T-75 (25 or 75 cm^2 surface area available for culture) to T-250, T-500 is considered a large-scale culture.
- Based on this perception, mammalian cells may be grown in several T-250 or T-500 for large-scale applications without additional capital investment and would be applicable as large-scale culture.
- ***However, in general for moderate or medium-level mammalian cell culture scaling up, the following instruments are used***:
  Spinner flasks
  Cell factories
  Roller bottle system
  Hollow fiber system
  Tabletop bioreactors
- Generally, the volume of the culture medium in the moderate level of scaling up is a few liters (e.g., the maximum volume of a spinner flask is 20 l).
- Medium-level scale-up instruments can be converted to a large-scale culture system. For example, nowadays a roller apparatus is available to accommodate **hundreds of bottles that may be utilized for vaccine production**.
- For large-scale mammalian cell culture, various **bioreactors are used**. Here, large-scale culture denotes >100 l of culture medium that may be as high as 10,000 l capacity.

  NB: For large-scale nonmammalian cell cultures, fermenters may be used; however, these are not the intent of discussion in this chapter.

  There are several objectives for large-scale mammalian cell culture. They are as discussed as following.

3 Objectives of Large-Scale Mammalian Cell Culture

- Mammalian cell culture begins after seeding fewer cell populations in a small cell culture apparatus (e.g., **a bench top 5 l bioreactor**). Once the cells grow, this small culture volume is subjected to the scale-up procedure.
- Scale-up is sometimes loosely used to attain an enhanced production capacity of a particular cell culture product after transferring the small volume of culturing material (**cell culture medium containing early to mid-log phase cells**) into a large cell culture apparatus (e.g., a **5000 l bioreactor**).

- Thus, in a large-scale culture, sophisticated mammalian cells grow in large numbers and a controlled environment within a large container.
- Scale-up may, therefore, be utilized as the "predictable" (**engineered**) increase in production capacity.
- **The primary objective of mammalian cell culture scale-up is to obtain high cell yield from as small a cell culture medium volume, as possible (for commercial benefit)**.
- The volume of the culture medium, as well as the cell population, is scaled up according to the need for cell culture products. For example, it can be 50 l, 100 l, or even 1000's l.
- Various instruments including bioreactors are generally used for scaling up mammalian cell cultures.
- In typical mammalian cell culture, homogeneity of the culture conditions can be maintained, allowing easier isolation of secreted cells and their important products (e.g., **vaccines, recombinant proteins, enzymes, antibodies (Abs)**) from the culture medium.
- Thus, the most important objective of large-scale mammalian cell culture is to obtain various commercial products in large quantities (Arathoon and Birch 1986; Hu and Aunins 1997; Bliem and Katinger 1988a, b; Broad et al. 1989; Ramasubramanyan and Venkatasubramanian 1990).
- However, there are several limitations of large-scale mammalian cell culture. ***The following paragraphs describe these factors in detail***.

4 Factors Affecting Large-Scale Mammalian Cell Culture

While the microorganisms are just single-cell living creatures, mammalian cells are a part of a tissue and organ of a multisystem complex body. Therefore, separating mammalian cells from the complex tissues and their subsequent culturing as single cells is highly challenging.

- As compared to microorganism size (e.g., bacteria: 0.2–0.5 μM), the mammalian cells are larger with 10–30 μM dimensions.
- As compared to the microorganism growth rate (e.g., *Escherichia coli*, a Gram-negative bacteria having a doubling time of just 20–30 min), the mammalian cells grow slowly (**doubling time** 10–50 h).
- While microorganisms are covered by a rigid cell wall, mammalian cells are covered by a delicate plasma membrane and the mammalian cell structure is maintained by the **cytoskeleton proteins**.
- The delicate mammalian cells are sensitive to rough handling that may **create shear stress**. ***In a mammalian cell culture system, the flow of the medium and the air circulation should be controlled and there should not be any generation of shear stress. Of note, shear stress damages the cells and severely affects the ultimate fate of the cells***.

- Mammalian cells are highly sensitive to **microbial contamination**. Since microorganisms grow much faster than mammalian cells and use up all nutrients rapidly, preventing microbial contamination is a major issue in mammalian cell culture. Thus, maintenance of sterility in mammalian cell culture is of utmost importance.
- Most of the constituents of the mammalian cell culture medium including fetal bovine serum (FBS) or fetal calf serum (FCS) are expensive. In the case of serum-free culture, costly serum constituents are supplemented.
- Except for some blood cells (e.g., B and T lymphocytes), all the mammalian cells are **adherent in nature** and therefore must attach to the culture containers for survival and growth. So, mammalian cells require specialized cell culture containers made up of polystyrene. However, for large-scale culture, mammalian cells could be **adapted to suspension cell culture**.
- During mammalian cell culture, many toxic metabolites (e.g., **ammonia, lactate**) are produced and product concentration (titer) is usually very low (μg ml^{-1}). **These toxic metabolites severely affect the culture environment, thereby mandating timid and efficient monitoring and periodic removal from the culture**.
- Finally, constant monitoring of temperature and pH is essential, since any change in optimal extents (pH of ~7.4 to 7.5 and temperature 37 °C) may severely affect the growth and productivity of the cells being cultured.
- A specially made CO_2 incubator or similar instrument with a constant ~5% CO_2 supply is highly essential for a small-scale culture of mammalian cells (McQueen et al. 1987; Lehmann et al. 1988; Leist et al. 1990; Kunas and Papoutsakis 1990; Kioukia et al. 1996; Schneider 1996; Croughan et al. 1989; Kimura and Wm 1996; Van der Pol and Tramper 1998; Chisti 2000).
- Figure 1 summarizes the above-mentioned hurdles in large-scale mammalian cell culture, where more than one factor may be the culprit at a given instant.

These are some important reasons which make mammalian cell culture a difficult process. The following paragraphs discuss these concerns.

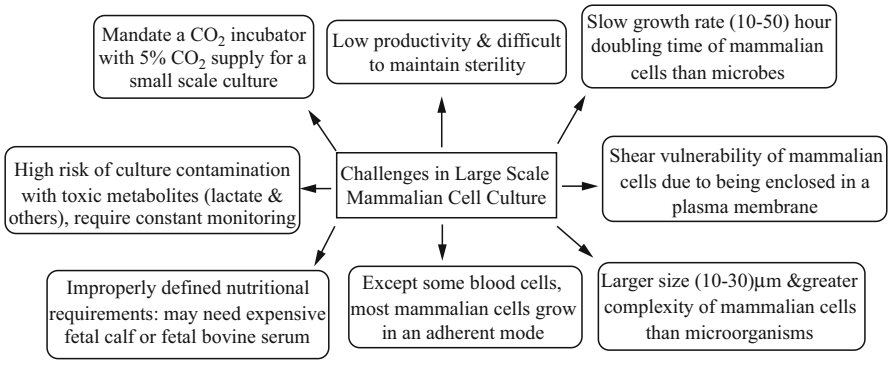

Fig. 1 Challenges associated with large-scale mammalian cell culture

5 The Scale-Up Operation of Mammalian Cell Culture

The following instruments or systems are used up for the scaling up of mammalian cell cultures:

1. Mammalian cell culture in spinner flask
2. Mammalian cell culture in cell factories
3. Mammalian cell culture in roller bottles system
4. Mammalian cell culture in hollow fiber system
5. Mammalian cell culture in bioreactors

Here is a brief discussion of the above instruments or systems.

5.1 Mammalian Cell Culture in Spinner Flask

- The spinner flasks are the most common and widely used large culture vessels due to their simplified setup and ability to provide a homogeneous environment throughout the mammalian cell culture process. By varying the agitation rate, the same type of spinner flasks may be used for various mammalian cell cultures.
- The **Bellco** spinner flasks are the most common and frequently used spinner flasks most probably due to their simplified impeller design.
- A spinner flask is made up of glass with a flat surface and a suspended central Teflon paddle. One or two side arms extending from the central region are used for sampling and decontamination (Fig. 2). While the minimum size of a spinner flask is as low as a few milliliters, the maximum is 20 l.
- The medium is aseptically inoculated with the mammalian cells, followed by placing the flasks on a magnetic stirrer. From the central region ring, the medium is mixed via stirring between 10 and 300 rpm. This stirring improves the gaseous exchange.
- In the original culture method, the cells in suspension mode are used.
- However, cell adsorbed scaffolds attached to needles are suspended from the top flask covered by the culture medium or cultured as adherent layers on microcarrier support.
- The suspended cells are not allowed to settle at the bottom of the flask.
- Spinner flasks move the culture medium contents with a unique oscillatory movement ensuring low shear forces and an efficient mixing.
- The homogeneous culture conditions in the spinner flask are generated at the expense of hydrodynamic forces introduced via the rotating impeller. Mechanical forces, such as shear stress, could alter the cell growth progress while a high shear environment could be damaging to the cells and the microcarriers.

Fig. 2 Front view of a Spinner flask. (Image incorporated from our laboratory workable setup)

The spinner flask system has the following advantages:

- During the stirring of the medium and in the entire cell culture duration, no heat generation takes place. Therefore, no heat is imparted to the cultured cell suspension.
- In most of the suspended or even adherent cultures, the cells maintain a high level of expression.
- It provides a high surface area to volume ratio, ensuring improved oxygenation, mimicking a traditional stirred tank reactor.
- Cultured constructs have higher seeding density and, thereby, allow a more uniform cell distribution compared to static cultures.
- Constructs of spinner flasks usually have a higher proliferation rate and cell content compared to those of static cultures.
- Since generally, a spinner flask of more than 20 l volume is not available, culture in a single spinner flask gives only a Low product quantity.
- For more information, readers should go through the relevant literature (Ismadi et al. 2014).

5.2 Mammalian Cell Culture in Cell Factories

- Cell factories are available in various formats.
- **Multitray or multiplate unit** is a good example of a cell factory.
- Multiple systems are polystyrene plates with multiple chambers. These plates are designed for single use (use and throw).
- Structurally a multitray system consists of 10 vertically connected fixed chambers. At the corner of the plates, interconnected channels are there which ensure a uniform distribution of medium throughout its addition at the top of the chamber. Readers are advised to refer https://www.ddbiolab.com/frontoffice/product?produitId=0A-32-03 to know about the cell culturing suited size configurations of cell factories (Fig. 3).
- The major use of culturing mammalian cells in cell factories is the establishment and subcultivation of cultures from human diploid working cell banks, for vaccine production.
- Exhibit varying volumes and surface areas.
- Used to inoculate similar microcarrier culture systems (Dinnis and James 2005; O'Callaghan and James 2008).

  NB: Various companies such as the NUNC cell factory system by ThermoFisher, supply multitray units. A detailed protocol is available on the company website.

5.2.1 Usefulness of Cell Factories

Multitray units are easy to use and give good results with large cell populations.

Limitations of Cell Factories

- It is a labor-intensive process.
- Single-use makes the culturing expensive.
- Trypsin-EDTA is used to detach the cells from the cell factories. However, all the cells cannot be washed out.

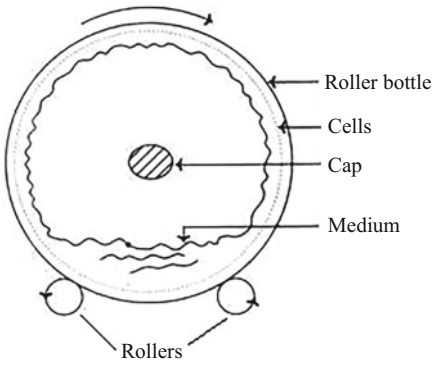

Fig. 3 Functional cell culturing probes of a roller bottle system

5.3 Mammalian Cell Culture in Roller Bottle System

A roller bottle system consists of several flasks fitted on a roller. These flasks can be filled with the cell culture medium. After the mammalian cells are inoculated, switching on the machine leads to rotation of the bottle system. On harvesting the cultured cells, the same flasks are further used for fresh cell culture (Berson and Friederichs 2008).

A variety of roller bottle systems have been developed starting from small bench tops, to cart-mounted or large automated ones that can accommodate standard-sized (~ 1050 cm^2) bottles.

- In the roller bottle system, the functional component of the apparatus used is called a **roller**, because it rolls or rotates. Readers are suggested to visit www.coleparker.in to have a better idea of cell culturing using the Roller Bottles System.
- ***A roller bottle system is a specialized kind of CO_2 incubator.***
- Each roller bottle has a **250 to 2000 ml capacity**.
- Nowadays, 4 to 100 bottles accommodating roller apparatuses are available. For example, the production of vaccines uses 30,000 bottles per batch.
- The roller is horizontally fitted with multiple cylindrical bottles.
- On being switched on, each roller bottle rolls around **its axis**.
- A roller bottle rotates at the rate of **(10 to 20) rotations per hour** while cells are attached.
- After cell attachment, a roller bottle system rotates at **(6 to 8) rotations per hour**.
- A uniform monolayer of cells at the inner surface of the bottles is usually observed.
- The entire cell monolayer is transiently exposed to the medium and air, on an alternate basis after each successive bottle rotation.
- The volume of the medium should be adequate sheerly for providing a shallow covering over the monolayer.
- The **spiral film method** and **glass tube method** may be used to further increase the surface area of roller bottles.
- In the roller bottle system, there is good control of the **gas phase**.
- However, **headspace volume** must be provisioned to maintain adequate oxygen and pH.

Usefulness of a Roller Bottle System

- Nearly all the internal surface in a roller bottle is available for cell growth. Thus, the **internal surface area is high enough to support cell growth**.
- In roller bottles, only **15–20%** of the **surface** is covered with the medium at a particular instant. A lower medium (concentration) with a smaller volume and high product titer could be obtained in this technique.
- **By rotating the bottle, the cells are alternatively subjected to the medium and air**. More importantly, the cells are **efficiently oxygenated** due to alternate exposure to the medium and the gas phase (Fig. 3).

- The **medium/air volume ratio is maintained between 1:5 and 1:10** to prevent oxygen scavenging.
- The medium is gently and constantly agitated so that there is no excess shear stress on the cells.
- Since the system is dynamic and rotates, it produces more cells than stationary cells.
- Collection of the supernatant medium is relatively easy in the roller bottles.
- Roller bottles can be reused and therefore their use is not much expensive.
- The major use of the roller bottle system pertains to the production of various proteins (e.g., erythropoietin) and vaccines (e.g., chickenpox and herpes zoster).

Limitations of Roller Bottle System

- Monitoring of cells in a large number of bottles is very difficult besides being labor-intensive.
- Skilled manpower is highly essential for cell seeding, medium change, harvesting, etc. of a large number of bottles.
- During handling a large number of roller bottles, chances of contamination would always be there.
- It is difficult to maintain the oxygen tension inside the roller bottles. Low oxygen incubators are cumbersome and expensive to run.
- The process involves multiple steps and is, thus, inefficient in terms of staff resources and materials.
- Since a large number of bottles are used for large-scale production, a large surface area (floor space) of the research laboratory is needed as working premises.
- Investment is rather high.

5.4 Mammalian Cell Culture in Hollow Fiber System

- In 1972, Richard and his group from the National Institute of Health (NIH), USA, used cellular acetate constituted of 1.5 cm^3 hollow fiber capillary membranes. When used for cell culture, these membranes formed 1 mm wide nodules in 28 days. Analysis revealed that from a starting cell bath of only **200,000 cells, 1.7×10^7 cells** could be harvested.
- The same research group further used polymeric and silicone polycarbonate constituted capillary membranes having less than 3 cm^3 volume and cultured human choriocarcinoma cells. Results revealed the expansion of existing cells to an approximate population of 2.17×10^8.
- The hollow fiber system was optimized to create a matrix analogous to the vascular system of a mammalian body.
- Structurally, acrylic polymer fibers of 350 mm diameter and a 70–75 mm thick wall are enclosed in a cartridge, with encapsulation at each terminal. Readers are suggested to refer to the 2016 contribution of Storm and colleagues (2016).

- So, a bundle of synthetic hollow fiber is used to fill up the vessel and **create a matrix similar to the vascular system**.
- The space between hollow fibers is known as an **external chamber**.
- For the growth of cells, the hollow fibers are perfused with a culture medium.
- So, technically, as soon as the fibers are packed, the medium is pumped through the lumen followed by perfusion through the fiber wall which is porous enough to passage the macromolecules having a molecular weight between 10,000 and 100,000 Daltons.
- The membranes of hollow fibers are semi-permeable. Thus, these membranes allow the diffusion of gases (oxygen), nutrients, and waste products albeit not proteins. The secreted proteins are retained in the extra capillary space, resulting in accumulation as high as 100 times greater than that of the conventional flasks or roller bottle system.
- Cells grow in the **extra fiber space**, exhibiting a tissue-like density.
- The nutrients are delivered from the bottom layer of cells and move upwards in a gradual manner as the cells are attached to the hollow fiber rather than a nonporous plastic dish.
- The cells are maintained for the continuous production of proteins for months even after being split (Hanak and Davis 1995; Storm et al. 2016).
- The product is harvested from the extra capillary space.

Usefulness of a Hollow Fiber System

- A very high surface area to culture volume ratio (till **30 $cm^2 \cdot cc^{-1}$**) could be achieved, allowing a large cell population density (up to **10^8/ml**) to be maintained.
- Hollow fiber cell culture is the only means for in vivo culturing of cells.

High-density cell culture has the following benefits:

- Reduced serum requirement and the adaptation to being a serum-free medium.
- 10 to 100 fold increment in the concentration of the secreted product.
- Viral and parasitic infections of mammalian cells proceed rapidly.
- In this system, cells get **good oxygenation and grow to high densities**.
- Cells are relatively easier to separate.
- This culture system is successfully applied to the large-scale expansion of cultured mesenchymal stem cells (**MSCs**).

Limitations of Hollow Fiber System

- It is difficult to remove cells from the system, particularly those which are adherent.
- The system majorly supports 2D culture and is unable to provide adequate spatial and metabolic support for stem cell culture (Fig. 4).

For a detailed protocol of the hollow fiber culture system, one can go through the reference: Storm et al. (2016).

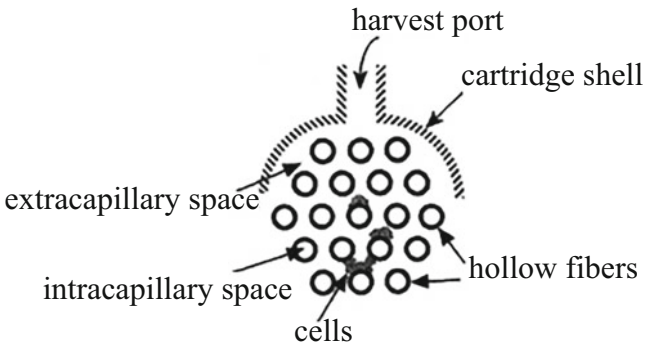

Fig. 4 Coordinated functional assembly of integrated fibers

6 Mammalian Cell Culture in Bioreactors

- A **bioreactor** is typically any device or system that supports a biologically active-controlled environment for culturing various cells including those of bacteria, fungus, yeast, and animals.
- Bioreactors are either single use (disposable) or multiple use.
- For the operation of a disposable small culture volume, single-use bioreactors are used.
- This will minimize the contamination of the culture medium.
- Using disposable bioreactors eases the requirement of cleaning and sterilization, maintenance, and validation of bioreactor runs.
- The disposable bioreactor runs can be scheduled for a closer operation, allowing an increased production.
- The stainless steel-constituted bioreactors are the predominant versions used for large-scale or industrial production of various protein products.
- These bioreactors mandate a validation for cleaning, sterilization, and maintenance, therefore requiring skilled manpower.
- Thus, these bioreactor systems need large-scale capital investment.

6.1 Relationship and Distinctions Between Mammalian Cell Culture Bioreactors and Fermentors

- In general, a bioreactor can be termed a **fermenter** if it is designed to support the industrial production of acids (e.g., lactic acid), alcohol (e.g., ethanol), or other products from bacterial, yeast, or fungal cultures.

- This implies that a fermenter is a **specialized bioreactor** predominantly used for **microbial cultures**, viz-*a*-viz multifold industrial requirements. The process is called **fermentation**, which could be anaerobic or aerobic. Thus, it is worth noting that a fermenter is not used to culture animal/mammalian cells.
- Animal cells are large and fragile (without any cell wall), grow much slower, and are, therefore, highly sensitive to shear stress. Thus, although conceptually a mammalian cell culture in a bioreactor and a fermenter is similar, there are several functional components based on distinctions between the two.

The following modifications are highly essential in a mammalian cell culture bioreactor:

- **A marine** (not turbine) **impeller** is necessary for mammalian cell culture. Impeller blades fitted at the end of the mechanical drive shaft are designed to allow vertical as well as horizontal liquid flow.
- A mammalian cell culture bioreactor must have **a curved** or **convex base** for better mixing of the medium at a slow but steady speed.
- Thus, a mammalian cell culture bioreactor must have a round bottom with a **2: 1 aspect ratio**.
- The **water-jacketed** (not immersion heater) temperature control is used in a mammalian cell culture bioreactor (Glaser 2000).

6.2 Design Requirements for a Mammalian Cell Culture Bioreactor

While designing a mammalian cell culture bioreactor, a wide range of parameters and possible functional aspects must be evaluated and screened upright, if a scale-up process is to be made successful (Varley and Birch 1999; Marks 2003; Abu-Absi et al. 2011; Whelan et al. 2012).

The following factors affect a large-scale mammalian cell culture:

- Mammalian cells are shear stress-sensitive, so the concerned bioreactor design should minimize the shear stress generation.
- Mammalian cells exhibit a slower growth rate (the growth rate of a mammalian cell cannot be changed, except for ensuring a steady nutrient supply for healthy growth).
- To counter the limitation of nutrients (e.g., growth factors) during mammalian cell growth, the bioreactor design should support a steady nutrient/medium availability and less resource-engaging product recovery and collection.
- Considering the hindrance mediated by lack of oxygen during rapid mammalian cell growth, a bioreactor design must ensure a steady supply of oxygen along with effective CO_2 removal.

- During the culture of mammalian cells, the accumulation of toxic metabolites (e.g., lactic acid) may affect the medium pH, so the configuration should ensure regular removal of toxic metabolites.
- Bioreactor design must resist the thermal fluctuations caused by changing medium temperature.
- Constant monitoring of temperature (37 °C), pH (7.4 to 7.5), moisture (95%), and elimination of toxic metabolites, all are highly essential in a commercial bioreactor design.
- Often there is an accumulation of dead cells caused by programmed cell death (apoptosis) during the cell culture. So a bioreactor design should facilitate easy removal of dead cells by standardizing the various factors (including culture duration) that could affect the cell health and physiology.
- For minimizing the contamination during culturing of slow-growing mammalian cells, the chosen bioreactor design should maintain sterility all through the culture duration.
- Nowadays, a large-scale culture system is available where everything is maintained robotically, without any human intervention.

Thus, an ideal bioreactor must have the following built-in characteristics:

Optimum structural design/configuration
Space for culturing adherent mammalian cells or cells in suspension
Controlled agitation
Adequate temperature control system
Mass transfer of fed substrate
Timely removal/collection of the culture products
Monitoring the pH, oxygen, and toxic metabolic by-products of cultured cells
Design for effective removal of toxic metabolites
Design for effective foam removal
Design for maintaining sterility all along the culture process

Here is a brief discussion of the above mammalian cell culture bioreactor design aspects:

6.2.1 Optimum Structural Design/Configuration

- A bioreactor should be designed in such a way that its construction is simple concerning measurements and control, compatible with up and downstream processing.
- Choosing the right bioreactor **configuration** is highly important for successful mammalian cell culture.

Design of power supply unit ensuring uninterrupted operation is also important for mammalian cell culture.

6.2.2 Space for Culturing Adherent Mammalian Cells or Cells in Suspension

- A bioreactor must have enough space for suspension of cells and adequate **surface area for adherent cell culture**.
- Finally, keeping a track of the inside environment (via sensors) is needed, whereby the changing circumstances (deleterious to growing cells) could be perceived (via computer software aided monitoring systems). This means that the software can tell us if conditions inside the bioreactor begin to deviate from the optimum so that corrective action could be taken to avoid an irreparable loss.
- Once it is detected that some parameters such as gas flow, fluid pump speed, or temperature controls are deviating from their standard values, compensatory adjustment is ensured via the bioreactors sensing provision.
- "Typical scale-up parameters such as power input per unit volume, kL_a (the mass transfer coefficient deciding the oxygen transfer rate), tip speed, and mixing times are defined with available data. This estimation assists the user in knowing about the input power requirement and the culture duration (to the optimum stage)."
- In summary, an ideal bioreactor may have a well-controlled environment including the standardization concerning **pH, temperature, dissolved O_2** and **dissolved CO_2 concentration, high cell**, and **product concentrations**, and **optimum medium** and **surface area utilization for adherent cell growth and scalability**.

6.2.3 Design for Controlled Agitation

- Several advances have been made in the large-scale unit process technology of a bioreactor that helps to overcome the multiple constraints of the traditional cell culture systems.
- In any bioreactor, the agitation of the culture medium is the basic step for regulating adequate nutrient and oxygen supply. Improper or high levels of agitation may generate shear forces that are injurious to sensitive mammalian cells. **Agitation and steady rate of gas transfer (O_2) ideally generate homogeneous conditions and ensure adequate nutrient, gas, and heat transfer rates**.
- Steady heat transfer is necessary during sterilization as well as temperature maintenance.
- Optimal mixing creates low, uniform shear. This must be ensured as a high shear force may damage the sensitive mammalian cells.
  Shear forces are affected by the following two design factors:

- **The shape of the impeller**
- **The speed of workable mode**
  Shear sensitivity of a mammalian cell culture depends on the following factors:

- Choice of the cell line
- Presence of key nutrients

- Concentration of inhibitory cell metabolites
- Batch age (cells are more sensitive to shear force in **lag** and **stationary phase**)

The shear sensitivity of mammalian cell cultures is of critical importance in the scale-up process. There may be restrictions on both stirring and sparging (rates) while scaling up the cell culture bioprocesses.

6.2.4 Design for Temperature Control System

- A bioreactor should be designed in such a way that there is no overheating of the culture medium.
- The primary heat transfer provisions in a bioreactor include **external jackets, internal coils**, and an **external surface heat exchanger**.
- **Generally, internal coils are not used in mammalian cell culture bioreactors** and are preferred only for a microbial culture supporting bioreactors.
- ***A temperature sensor should always be used to monitor the temperature of the inside environment of a bioreactor***.

6.2.5 Design for Optimum Mass Transfer of Feeding Substrate or Culture Products

Feeding substrate with adequate mass transfer (**prevention of under or overdosing**) and culture product transfer is essential for ensuring a proper and well-defined flow condition. There must be a ready and automatic monitoring of nutrient supply (e.g., glucose and L-glutamine), the two main energy sources of mammalian cells.

NB: The online flow injection analysis (FIA) using gas chromatography (GC), high-performance liquid chromatography (HPLC), etc. is used to monitor glucose, glutamate, and ammonia.

6.2.6 Design for Monitoring pH, Oxygen, and Toxic Metabolic By-products of the Cultured Cells

- The mammalian cell culture medium maintains a pH of around 7.4–7.5.
- As the mammalian cells grow and metabolize, there is an accumulation of toxic metabolites (e.g., lactic acid, ammonia), which may change the medium pH. So, there should be adequate provision for the periodic removal of generated wastes from a bioreactor. A bioreactor should be designed in such a way that it can monitor the pH of the culture medium being processed. In fact, in a bioreactor, **a pH sensor is utilized for on-time monitoring of medium pH**.
- **A pH control solution may be added as a remedy**.
- There must be an adequate and proper oxygen supply for the cultured cells. The bioreactor should have the provision to check the oxygen level via an **oxygen sensor**. **Higher cell densities generally increase the oxygen demand**.
- There must be minimum or no bubble generation since **bubbles may damage the sensitive mammalian cells. Compressed air entering a bioreactor is usually devoid of moisture and any sort of oil vapors that may originate from the compressor**.

- There should be an adequate provision to ascertain the extent of **toxic metabolic byproducts such as lactic acid, ammonia**, external contaminants, and impurities that may affect the culture condition.
- In course of mammalian cell culture, some cells die either due to programmed cell death (**apoptosis**) or because of a **stressful internal environment** (**necrosis**). However, the number of dying cells must be monitored since a high cell death severely affects the further generation of the cells. Therefore, monitoring the number of dying cells is important. Checking the relative extent of **lactate dehydrogenase** may aid in distinguishing the living and dead cells.
- Limitless culture duration could be attained using the fed-batch culture mode.

6.2.7 Design for Effective Removal of Toxic Metabolites

- Recently fabricated automated bioreactor systems have inbuilt perfusion accessories, thereby minimizing the risk of toxic metabolic product accumulation (e.g., **lactic acid from glucose and ammonia from glutamine**).
- Studies have shown that as the scale of the process operation increases, so does the toxic metabolite concentration.
- This increase in animal cell culture process duration (as needed in the scale-up operation) may lead to CO_2 accumulation. Such escalations in CO_2 can eventually reach metabolism-altering levels, as also reported by Kimura and Millar, in their observations of cultured Chinese Hamster Ovary (CHO) cells.
- The removal of such waste metabolites using **fed-batch/perfusion culture** should be performed during cell cultivation.

6.2.8 Design for Effective Foam Removal from Bioreactor

- During agitation of cell culture medium or supply of oxygen, accidental foam generation may damage the mammalian cells.
- To resolve this, a mechanical foam breaker (**a supplementary impeller**) is installed in a bioreactor.
- Alternatively, **chemical antifoam agents** (reducing oxygen transfer rate) could also be used.

6.2.9 Design for Maintaining Sterility All Along the Culture Process in a Bioreactor

- Inside a bioreactor, sterility must be maintained from the beginning of the culture to the end stage, corresponding to every stage of mammalian cell culture processing.
- So, to ensure a sterile environment in the bioreactor and minimize the contamination risk, gases introduced in the bioreactors should be eliminated through **a sterile filter**.

Large-scale bioreactors are presently controlled by robotics at every culture stage, minimizing human exposure and contamination chances.

6.3 Major Structural Components of a Mammalian Cell Culture Bioreactor

6.3.1 A Typical Bioreactor Consists of the Following Bodyparts

The medium-scale bioreactor (e.g., up to 20 l capacity) is made up of glass with a stainless steel head plate. In larger bioreactors, the head is rather entirely made up of stainless steel.

6.3.2 Agitator

It is used for mixing the reactor contents during which the cells are kept in a perfect homogenous condition for a more homogeneous nutrient and oxygen transport to the desired product(s) (Table 1).

6.3.3 Baffles

Baffles are the typical obstructing vertically arranged vanes or elongated plates inside the bioreactor needed to stop the radial swirl inside a **bioreactor** and convert the rotational flow to axial mixing. It is used to inhibit the vortex formation in the vessel, normally undesirable as it changes the system's center of gravity besides consuming additional power.

6.3.4 Sparger

The ring **sparger** is a **sparger** pipe equipped with ring-supported installation in the culture vessel lid. The ring has small perforations, which when connected to pressurized air, distribute it as fine bubbles (in the bioreactor/fermenter), allowing effective aeration. In the aerobic cultivation process, the purpose of the sparger is to supply adequate oxygen to the growing cells. Sparging refers to the process of injecting a gas through a diffuser into a liquid phase.

6.3.5 Jacket

- A **jacket** is a cavity external to the **culture-holding bioreactor vessel** that permits the uniform exchange of heat between the fluid circulating in it and the vessel walls. The jacket provides an annular area for water circulation at a constant temperature, keeping the bioreactor temperature as constant.
- Bioreactors often require a remote sensor, located within the culture medium. Its typical function is to **control temperature**. The chiller can, therefore, sense and **control** the internal environment and accurately maintain optimal conditions.

Table 1 Working aspects of agitator: Agitator speed and impeller diameter

Agitator	Agitator speed	Impeller diameter
Flat Blade Turbine	(100–300) RPM	1/3rd of tank diameter
Backward Blade Turbine	(100–300) RPM	1/3rd of tank diameter
Paddle	50 RPM	80% of tank diameter
Anchor	50 RPM	80% of tank diameter

RPM rotations per minute

NB: Besides reusable bioreactors, recently single-use bioreactors have also found much liking. Single-use bioreactors are more of a choice because of the null contamination risk from the previously cultured batch.

6.4 Types of Bioreactors for Mammalian Cell Culture

The following are the major bioreactor types:

1. Stirred tank bioreactors
2. Membrane stirred tank bioreactor
3. Spin filter stirred tank bioreactor
4. Bioreactors with bubble column
5. Airlift bioreactors
6. Tower bioreactors
7. Bioreactors with fluidized bed
8. Packed bed bioreactors
9. Photo-bioreactor
10. Vibromixer

Here is a brief discussion of the various bioreactors:

6.4.1 Stirred Tank Bioreactors

- **Stirred tank bioreactors** (STBRs) are **most widely employed** for culturing mammalian cells to harvest **enzymes, Abs, or other agents of similar grade**. These bioreactors enable an efficient mixing of the various phases via internal mechanical agitation.
- In the recent past, STBRs have been commercially developed for large-scale mammalian cell cultures that can handle **10,000 l** or even more.
- For laboratory use too, there are numerous **bench-top STBRs**, having **1–5 l** capacity besides a commercial availability for mammalian cell cultures. Some companies produce small STBR with **20 l** capacity.
- Thus, in the STBRs, either **small flasks** or **long vessels** are used. In both cases, **a stirrer is used for mechanical agitation which aids in aeration**.
- While the bench top STBRs are **made up of glass** with **a stainless steel head plate**, a large STBR is entirely made up of **stainless steel.**
- This bioreactor is fitted with a magnetized rotating pendulum and two side arms, one for the cell and medium addition while the other for gas supply.
- The stirrer is **moved by a motor** situated at the top of the vessel. It allows better and more efficient mixing control besides providing a relatively homogenous culture environment.
- The cultured cells are periodically exposed to the impeller discharge flow, at short time intervals.
- A cylindrical vessel with a motor-driven central shaft **forms a continuous STBR**. One or more **impellers** or **agitators** are supported by a continuous STBR.

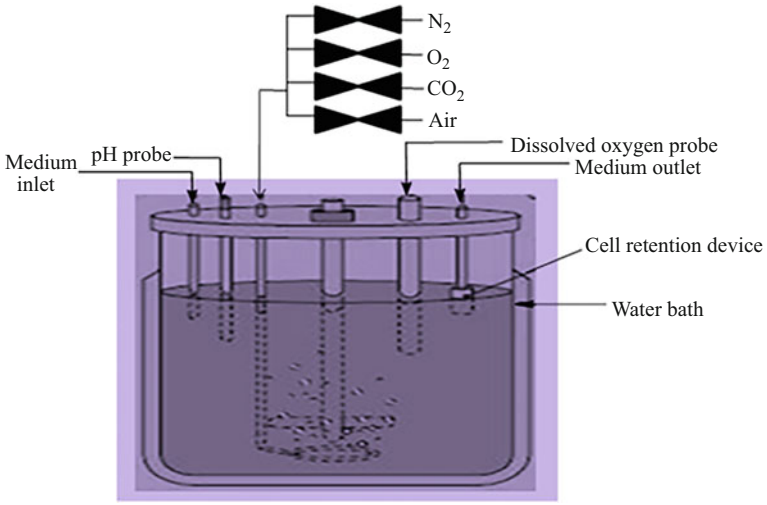

Fig. 5 The operational mode of a Stirred tank bioreactor. (Figure incorporated from Jäger et al. 1988 with modification)

- The shaft is fitted at the bottom of the bioreactor. The number of impellers is variable, depending on the size and several other factors such as **height to diameter ratio, referred to as aspect ratio (AR)** (Fig. 5).
- To know about the different impeller configurations, readers are suggested to refer to the literature sources, Vorlop and Lehmann (1989) and Chisti (1993).
- **The AR of an STBR for mammalian cell culture is usually <2**.
- The impeller diameter is usually one-third **of the vessel diameter**.
- The two impeller's distance is approximately 1.2 times the impeller diameter.
- In STBRs, the air is added to the culture medium under pressure through a device **called sparger**.
- The sparger may be a ring with many perforations or a tube with a single orifice. The major function of a sparger is to enable a homogeneous distribution of gas throughout the vessel, accomplished using **impellers** (**agitators**).
- The impellers disperse the air bubbles generated by the spargers into smaller ones. Subsequently, these small air bubbles create a uniform and homogenous environment throughout the bioreactor (Jäger et al. 1988; Vorlop and Lehmann 1989; Chisti 1993; Woodside et al. 1998).

  There are five types of impellers/agitators configurations in use, which are as follows:

- Marine blade
- Pitched blade
- Spin filter
- Draft tube
- Double screen cell lift

- Temperature is controlled by a heating pad or jacketed water at a constant value of 37 °C.
- Finally, there are several features of STBRs that enable **adequate control of culture parameters such as agitation, temperature, pH, and oxygen supply**.

Advantages of Stirred Tank Bioreactors

1. Commercial availability
2. Flexible operating conditions
3. Versatility and efficient gas transfer to the growing cells
4. Efficient pH control
5. Bench/tabletop capacity (5–20 L)
6. Increased capacity (100–10,000 L)
7. Easy to use and process
8. Less expensive method
9. Allow precise temperature control
10. Simple to control needed sterile condition
11. Easy culture of suspension cells
12. Also, allow adherent cell culture

Disadvantages of Stirred Tank Bioreactors

1. This kind of bioreactor needs shaft seals and bearings.
2. The weight of the bioreactor is unexpectedly high.
3. Mismatching length of the shaft concerning bioreactor functioning.
4. Usage is restricted to lab-scale due to the large requirement of cell-entrapped beads.

Applications of Stirred Tank Bioreactors

- In an STBR operation, reduced labor is required.
- STBRs are important for biomass production.
- STBRs find extensive use in pharmaceutical industries and for large-scale metabolite production.

NB: "Several agitation configurations, for example, turbine-type or marine-type impeller, vibromixer, and cell lift, have been developed to prevent high shear exposure of cultured animal cells. In large-scale or high cell density perfusion cultures, air sparging or oxygen-permeable silicone/polypropylene tubing provides adequate aeration."

6.4.2 Membrane Stirred Tank Bioreactor

- This bioreactor was developed by ***Professor Jvrgen Lehmann*** in the 1980s.
- It utilizes long macroporous polypropylene tubing wrapped around rotating rods.
- To facilitate direct gas contact with the medium, the micropores expand *by* adjusting the air pressure in propylene tubing (this provision ensures a gas sparger working *by* preventing the bursting).
- The tubing rotation provides a gentle agitation to microcarriers/suspended cells.

Distinction of Membrane Stirred Tank Bioreactor
Regulated tubing action avoids foaming, even at high serum concentration.

6.4.3 Spin Filter Stirred Tank

- This bioreactor works by regulating the movement using a central wire cage, mounted on an agitation shaft.
- The cage bottom is solid, whereas the side is made up of a wire screen having a 25–60 μm range opening (Fig. 6).
- The average cell diameter acquired ranges ~10–15 μm.
- The fresh medium needs to be continuously added from outside the draft tube, while the culture fluid is withdrawn from within the cage at the same rate.
- Some specific operational deviations may lead to lower cell concentration inside the stirring cage compared to the outside, leading to cell retention in a bioreactor.
- In this bioreactor, the cell concentration could be as high as ten times that of a typical STBR. The retention of such a high cell density is not due to centrifugal force (exerted by rotational cage motion), since the terminal velocity of cells due to centrifugal force is 2 to 3 times lower than fluid velocity.
- Owing to the high ionic strength of the culture field and low thickness of the Debye layer (typically 1 nm), the electrostatic effect also seems unlikely to retain such a large cell density.
- The effectiveness of particle retention is indexed by the discharge factor, with a value of 1 indicating null retention.

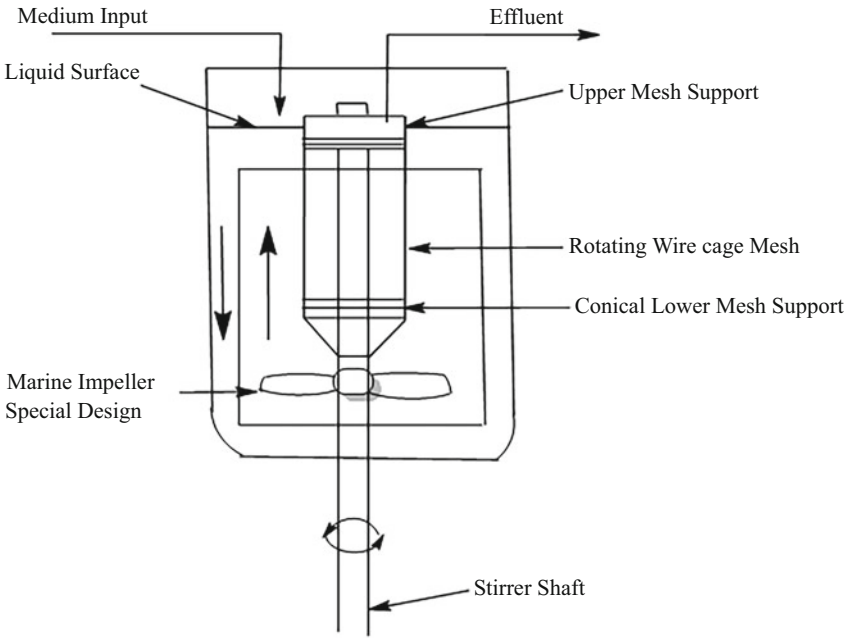

Fig. 6 Components of a spin filter stirred tank

- Studies on variations in this factor under different operating conditions, using polystyrene particles of similar diameter and density (as of cells), revealed its dependence on agitation rate.
- It was noted that an increase in the agitation rate from 50 to 100 rpm decreased the discharge factor after which further increment in agitation rate increased the discharge factor. So, cell retention is maximum within an optimal discharge factor range.
- An increase in agitation rate increases the discharge factor (after an optimal range), further assuring that merely centrifugation is not a responsible factor for cell retention.

Advantages of Spin Filter Stirred Tank Bioreactor

- Besides simple suspension cultures, the caged rotating wire provision in a spin filter stirred tank facilitates aggregation in microcarrier cultures.
- Smaller pore opening on the screen than most of the aggregate sizes enables a much simpler way of cell retention.
- The micrometer ranged diameters of most microcarriers or aggregates is the reason for centrifugation-driven particle retention.
- Rotating wire cage confers a unique ability to retain single suspension cells, presumably due to fluid mechanical effect, similar to the motion of **low Reynolds number particles** near the wall in a laminar boundary layered flow.
- Once the optimum working conditions are set up, the wire cage provision in a spin filter stirred tank allows an efficient scale-up of mammalian cell cultivation.

Disadvantages of Spin Filter Stirred Tank Bioreactor

- Cumbersome design and operation along with multiple control factors affecting the discharge extent often complicate the accurate prediction under different working conditions.
- The flow pattern in the cage vicinity is complicated, exhibiting a rotational regime caused by cage rotation and the perfusion-driven perpendicular flow along the inward direction to the cage.

6.4.4 Bubble Column Bioreactors

- The terminology bubble column bioreactor (BCBR) arises from the bubbling air that is introduced into the cell culture medium for aeration.

Gas distributors used in BCBRs include the following:

- Single-orifice nozzles
- Membrane or ring-type distributors
- Sintered, perforated, or porous plates
- ARM spargers
- Various hydrodynamic parameters need to be optimized to design ideal BCBRs. The parameters that need attention include specific gas-liquid interfacial area, **Sauter** means bubble diameter, overall heat transfer coefficient between slurry

and immersed heat transfer assisting materials, mass transfer coefficients for all constituents, gas hold-up times, and physiological properties of the liquid medium.

- The BCBRs vessels are cylindrical in shape and the aspect ratio is 4 to 6.
- In this bioreactor, the sole source of agitation is the pneumatic power generated by the isothermal expansion of the gas that is to be sparged. The scenario leads to an average shear rate that is an exclusive function of superficial gas velocity and the rheological parameters of the fluid in a manner that is analogous to autonomous theoretical scrutiny.
- The perforated plates may be used to fit the BCBRs.
- The input air or gas is injected across the column base via an orifice possessing pipes/plates or microporous metal spargers as described earlier (Fig. 7).
- Typical gas or air flow rates affects the performance affecting parameters, such as oxygen transfer and mixing.
- Generally, shear stress-sensitive mammalian cells are not suitable for being cultured in the BCBRs (Katinger et al. 1979; Romer 1979).
- Readers are suggested to consult the 1979 publication of Katinger and colleagues, to get more acquainted with the integrated functional setup of a BCBR assembly, comprising of temperature-controlled medium reservoir and product collector.

6.4.5 Airlift Bioreactors

- These bioreactors are specialized for the distribution of vessel medium into two interconnected zones, separated using a baffle or draft tube (Fig. 8).
- **The riser**: It is one of the two zones where air/gas is pumped.
- **The downcomer**: The other zone where there is no air or gas.
- The aerial sparging domain of the reactor.

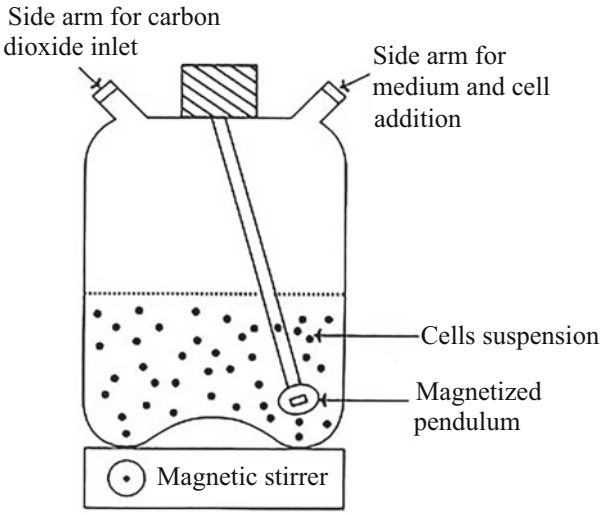

Fig. 7 The typical working framework of a Bubble Column Bioreactor, main reactor container

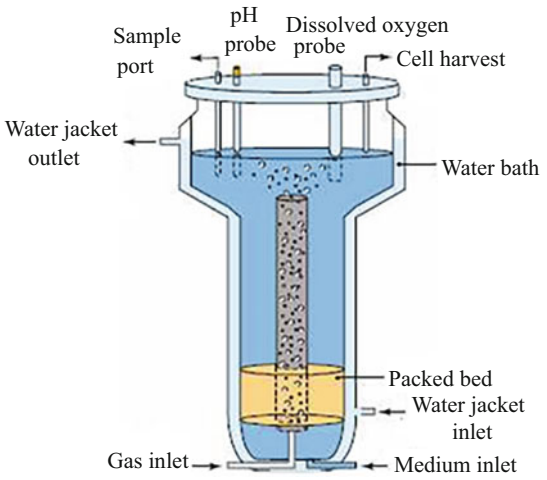

Fig. 8 Components of an airlift bioreactor. (Original figure adapted from Chisti 1989, with modification)

- The upward circulation of liquid is possible because of the difference in hydrostatic pressure between the **spacer section** (lower effective density) and the **bubble-free section**.
- There are two types of airlift bioreactors (**ABRs**).

6.4.6 Internal Loop Airlift Bioreactors

- In these simply designed bioreactors, the volume and circulation rate are optimized/fixed for fermentation.
- In this type of bioreactor, the **cell culture medium and the air circulate together**.
- To create interior liquid circulation, channels of this single container along a central draft tube are utilized.

6.4.7 External Loop Airlift Bioreactors

- An external loop is present in this bioreactor.
- Thus, the cell culture medium circulates through separate independent channels.
- However, to meet the requirements of different reactors, these reactors can be modified.
- In general, the ABRs are more efficient than BCBRs, enabling the processing of denser cell suspensions. This is mainly because of better content mixing in these bioreactors compared to BCBRs (Chisti 1989).

Advantages of Airlift Bioreactors

- The ABRs work based on aerobic bioprocessing technology.
- "**These reactors ensure a pumping assisted controlled liquid flow in a recycle system**."

- In the biotechnological domain, these bioreactors are recognized as highly efficient instruments for the production of various molecules including single-cell protein, and methanol besides integrated waste management.
- The air pumping (injection) and the liquid circulation determine the performance of ABRs.
- ABR is suitable for the culture of fragile mammalian and plant cells.
- ABRs are befitting low shear devices due to null mechanical agitation.
- These bioreactors employ a direct mode of air sparging which does not cause excessive cell damage to mammalian cells.
- Bubble size can be controlled by varying the sparger type.
- These bioreactors are not much expensive because of their simpler mechanical configurations.

Disadvantages of Airlift Bioreactors

- Considerable back-mixing in both gas and liquid phases often results in a high-pressure drop and bubble coalescence.
- Although simpler in construction, design features are critical in maintaining optimal hydrodynamic behavior.

Applications of Airlift Bioreactors

- Successfully used for BHK-21 cells suspension cultures, human lymphoblastoid cells, CHO cells, and hybridomas.
- Highly efficient for mass transfer.
- The flow and the mixing properties of vessels are improved by this bioreactor.
- The temperature-dependent product formation used two-stage bioreactors.
- Cells first grow at 30 °C and are then pumped into another bioreactor having a temperature of 42 °C.
- Each bioreactor of a two-stage ABRs is fitted with valves connected by a pump and a transfer tube (Fig. 9).
- While the first bioreactor is used to grow the cells, the second one is utilized for bio-processing.

6.4.8 Tower Bioreactors

- A tower bioreactor constitutes a large dimension pressure-cycle fermenter.
- In this bioreactor, high hydrostatic pressure is generated at the bottom of the bioreactor, which increases the medium solubility of O_2 and allows high aeration capacity.
- To complete the cycle, the medium flows down in the down corner.
- These bioreactors are predominantly used for microbial culture and are not preferred for mammalian cell culture.

6.4.9 Fluidized Bed Bioreactors

- Fluidized bed bioreactors (**FBBR**) are comparable to BCBRs with the only difference being the expanded top position that reduces the fluid velocity.

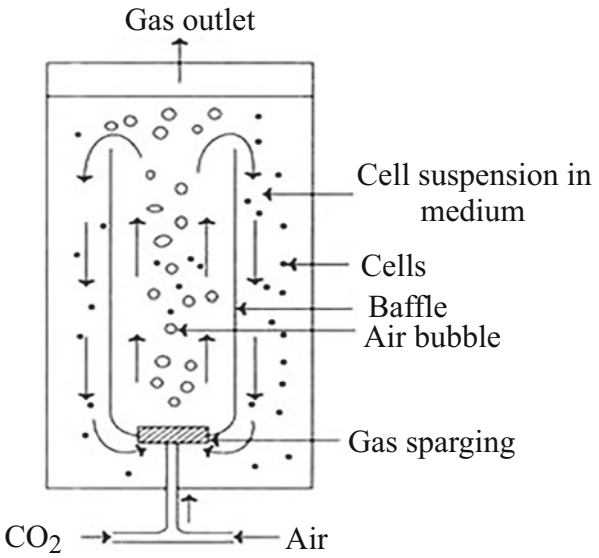

Fig. 9 Pictorial depiction of a two-stage airlift bioreactor

- The design of the FBBR (expanded top and narrow reaction column) is optimized to retain solids and facilitate liquid outflow (Fig. 10).
- These bioreactors are capable of conducting reactions that involve fluid-swinging biocatalysts. Eminent examples comprise immobilized enzymes, cells, and microbial flocculants.
- Usually, a gas is injected to establish an appropriate gas-liquid-solid fluidized bed, for the optimum functioning of fluidized beds.
- It is further essential to note that suspended solid particles should have an optimum density (contrary to low resulting in floatation while a higher extent resulting in sedimentation) so that the constituents remain thoroughly hanging up mode.
- The technology herein recycles the moving fluid which enables continuous contact between the enzymes and reaction constituents.
- Such an operational mode ensures a regulated functioning with high bio-processing efficacy.
- Predominantly used for **microbial** culture. However, mammalian cells are also cultured in FBBRs (Reiter et al. 1991; Detzel et al. 2010).

6.4.10 Packed Bed Bioreactors

- These reactors are characterized by a bed constituted of solid particles, having enzymes residing on or within the solid matrix, enclosed within a column.
- The solid materials suitable herein could be porous or nonporous gels and could be compressible or rigid.

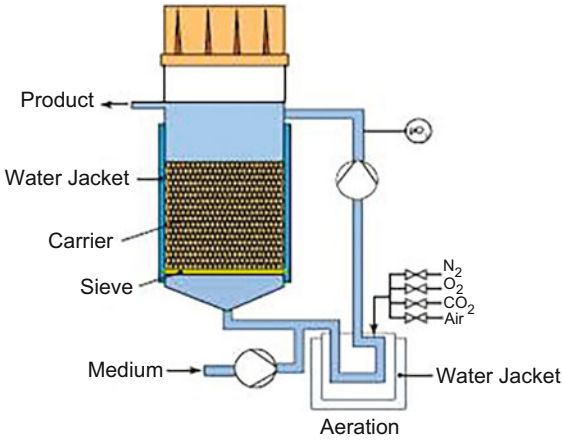

Fig. 10 The working framework of a fluidized bed bioreactor. (Original figure adapted from Reiter et al. 1991 with modification)

- Working configuration is characterized by a continuous flow of nutrient broth over the immobilized enzyme.
- Bio-products formed in PBBR (during culturing live cells) are discharged into the liquid culture medium and are gradually extracted by adjusting the upward and downward movement of the culture medium (normally, downward movement is chosen under the influence of gravity).
- By increasing the nutrient broth in flow, the concentration of nutrients (in the culture medium) as well as the extent of products formed could be increased.
- Due to inappropriate mixing, it is quite cumbersome to adjust the PBBR pH via adding acid or alkali.
- Despite several performance constraints, these bioreactors are used for the sustained conduct of feedback reactions.
- The distinction between these bioreactors' functioning is their ability to disallow an accumulation of formed bio-products.
- These bioreactors are not the first line choice for mammalian cell culture (Chunshui Cong et al. 2001; Meuwly et al. 2007).

6.4.11 Photo-Bioreactors

- These bioreactors are specialized for fermentations achievable either via **exposure to sunlight or artificial illumination**.
- These systems are suitable for the production of microbial products outside their natural environment.
- Preferred especially for the cultivation of phototropic organisms (those growing using light energy).
- Cultured microorganisms use photosynthesis to build their mass using light and CO_2.
- Familiar examples include plants, mosses, microalgae, cyanobacteria, and purple bacteria.

- These reactors provide an environment suited for higher growth and purity rates than provided by natural habitats.
- **P-carotene** and **astaxanthin** are some important compounds produced using photo-bioreactors.
- These bioreactors are not suitable for mammalian cell culture.

6.4.12 Vibromixer

- Vibromixer uses a perforated disk for mixing in and also finds utility as a parallel conventional impeller.
- The disk vibrates at a very high frequency in the vertical direction, causing the liquid to circulate through the perforations and facilitate mixing (Fig. 11).
- Have been used since the 1960s for the cultivation of suspension cells and virus production.
- Vibromixer is used to keep concentrated microcarriers in suspension mode for cell detachment during trypsinization.
- Owing to scale-up difficulty, these bioreactors have not been used for cell cultivation in the past few decades.

6.5 Selecting a Particular Bioreactor to Culture Mammalian Cells

- Since there are several bioreactors available to culture mammalian cells, sometimes it becomes difficult to make an optimum choice for a specific purpose.
- The selection majorly depends on the amount of product (mainly proteins) needed for the bioreactors. The requirement of proteins may be in mg extent (for laboratory research purposes) or sometimes in a few kg (industrial scale).
- For laboratory-scale, small disposable single-use bioreactors or flasks, membranes, or bag systems are used.
- For the small-scale culture, the fixed bed, fluidized bed small bioreactors, and small tabletop single-use disposable bioreactors (up to 5-l culture) can be used.

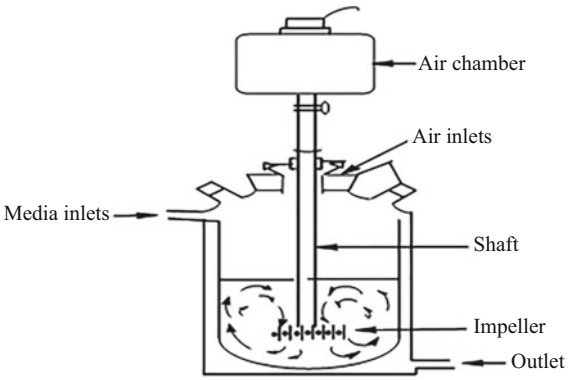

Fig. 11 Assembly of a vibromixer

- In small reactors, suspension culture is preferred over fixed bed culture.
- For larger product quantities, only suspension reactors or up to a certain scale, FBBR provides the required feasibility. In this case, STBRs may be the first line of choice.
- Generally, chemostat bioreactors are used for continuous culture of mammalian cells.
- Airlift bioreactors have been successfully used for suspension cultures of BHK-21 cells, human lymphoblastoid cells, CHO cells, and hybridomas.
- "While selecting the cell culture bioreactors, special requirements should be considered, such as gentle agitation and aeration without cell damage, a well-controlled environment concerning pH, temperature, dissolved oxygen, dissolved CO_2 concentration, *etc*., low levels of toxic metabolites (ammonia, lactate, etc.), high cell and product concentrations, optimized medium utilization, adequate surface area for adherent cell growth and scalability."
- Thus, a large number of utilizable mammalian cells could be/should be grown using a minimum quantity of cell culture medium to moderate the cost of the specific product being produced by the mammalian cells.
- The development of automated bioreactors allows an effective optimization and monitoring of various parameters such as temperature, pH, and aeration.

  Large-scale bioreactors are now using robotics for all the functions with minimum or no exposure to humans, thereby minimizing the contamination.

- To protect the mammalian cells from exposure to varying physiological environments, including altering pressures and shear forces, some essential additives may be added to the culture medium.
- Now, a lot of limitations in the large-scale mammalian cells culture have been overcome by standardizing various protocols, and a large number of proteins, monoclonal antibodies (MAbs), and gene therapy products are produced to significant extents (Barradas et al. 2012).

6.6 Knowledge Requirement to Work on a Bioreactor

To work on a bioreactor, the following knowledge related to biomedical and bioprocess engineering is highly essential (Hutmacher and Singh 2008; Burova et al. 2019).

- Reaction kinetics
- Mass and energy balances within a bioreactor
- Mixing kinetics
- Understanding mass transfer and heat transfer

The Use of Computational Fluid Dynamics Software

- Several parameters about the use of bioreactors can be calculated using computational fluid dynamics (**CFD**). This software can optimize a bioreactor design and the flow conditions (Bilgen et al. 2005). Some of the parameters that are successfully calculated by using this software include flow fields, shear stress, and mass transport within and in the vicinity of 3D tissue preparations. The other important applications of this software are regulating the O_2 distribution in a rotating-wall bioreactor, modeling direct perfusion in different scaffold configurations, and evaluating the effect of pore structure and interconnectivity on tissue maturation (Martin et al. 2004).
- Besides the CFD, some more modeling tools are currently in use. For instance, computed tomography scanning can be used to construct computer-based models of tissue-engineered scaffolds (Cioffi et al. 2008).

  NB: For a research protocol regarding culturing the cells in a rotating wall bioreactor, readers are suggested to go through the Radtke and colleagues work (Radtke and Herbst-Kalovetz 2012).

6.7 Scale-Up Operation in a Bioreactor

- The major objectives of scaling up the bioreactor-mediated mammalian cell culture are to ensure adequate growth of desired cells so that the maximum amount of various commercially significant cell-based products can be isolated.
- For the scale-up of the culture, all the parameters related to cell culture must be optimized.
- In this case, product cost should be the least while the quality and standard of the specific product(s) must be maintained to the highest.

  For process optimization, the following parameters must be considered:

  The type of cell being used.
  The type and composition of the **right nutrient medium**.
  The feed and operational settings to get the maximum amount of product(s).
  Minimizing the byproduct generation (if any).

- Generally use of serum in the cell culture medium is avoided since it not only increases the product cost but also enhances the likely contamination by mycoplasma, viruses, etc. So, supplements of important serum constituents must be standardized for the large-scale mammalian cell culture.
- The typical process generally consumes 3–4 weeks depending on the scale and complexity.
- Ideally, one believes to spare at least **2–4 months** in optimizing a proven scale-up operation to get a fast and lean transition from small to production scale.
- During the scale-up process of a mammalian cell culture facility, all the cells must be maintained in **similar conditions with steady-state growth**.

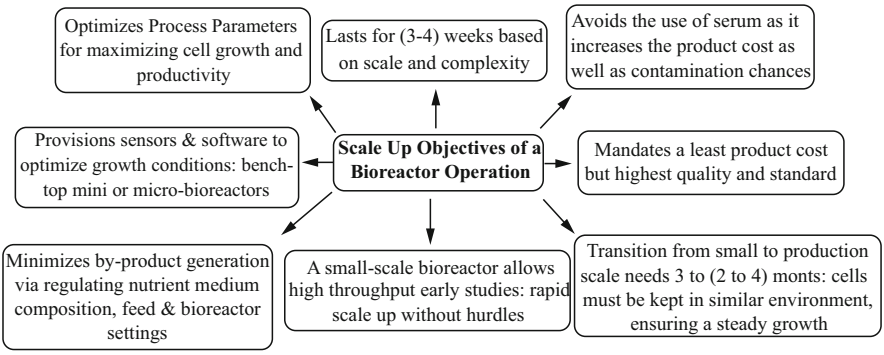

Fig. 12 Scale-up objectives of a bioreactor

- At present several bench top mini- and micro-bioreactors are available. These bioreactors are incorporated with various sensors and other software that help in the standardization of all the working parameters at the initial level.
- Towards this direction, initial optimization for working on a **small-scale bioreactor** is recommended. Thereafter, switching over to main bioreactors "enables high-throughput early studies" and allows a rapid process scale-up without problems.
- Finally, once again no contamination at each culture stage must be the objective of every bioreactor cultured mammalian cell.
- Figure 12 summarizes the scale-up objectives of a bioreactor for an easier understanding. It must be noted that meeting all requirements simultaneously is challenging, but the relationship between optimized parameters is intricate, so a parameter having a greater significance concerning production control must be optimized earlier.

6.7.1 Operating Mode of Bioreactors

The first step of bioreactor operation comprises inoculation with the cultured cells.

After inoculation of the cells, a bioreactor could be operated in either of the following three modes:

Batch culture
Continuous culture
Fed-batch culture

Here is a very brief discussion about them:

Batch Culture

This mode of culture requires no addition of new medium or nutrients after its commencement. After completion of the culture process and waste removal, the formed products could be collected. Thereafter, a new culture batch may be started if or when it is necessary.

Continuous Culture

In this mode of bioreactor operation, the culture process is continued until the required product quantity is not obtained. To accomplish this, the continuous addition of nutrients, medium, etc., and continuous harvesting of the product (s) should be done. There may be significant chances of contamination.

Fed-Batch Culture

This is the most common type of culture mode in a bioreactor. In this mode, the culture process begins with a low media amount. Thereafter, feed nutrients and medium are added as per an optimized program with no harvesting of generated bio-products.

NB: On completion of the culture process, the formed product(s) is harvested by the downstream operation.

The following paragraphs describe the working process of the batch, fed-batch culture, and continuous flow or perfusion cultures:

1. **Batch/fed-batch culture**
2. **Continuous flow or perfusion culture**

Here is a brief discussion on the operational modes of bioreactors.

1. **Batch and Fed-Batch Culture**

The batch culture starts with **an initial, limited amount of nutrients in a limited volume** (Zhou et al. 1995).

- Inoculated culture passes through several phases (***lag, log, stationary, and death***) following a growth curve.
- In this culturing mode, as the cells grow older and near their death, the nutrients are consumed and metabolic by-products gradually accumulate.
- Cells are harvested before the death phase, *that is*, either at the late log phase or the beginning of the stationary phase.
- **No medium component or inoculum is added during the cell culture duration, so nutrients are consumed as living cells take them up** (Fig. 13).
- Batch culture is generally used only for small-scale purposes.

NB: Generally, STBRs are used for mammalian cell batch culture growth.

- To **prolong a batch culture**, substrate feeding at gradual time intervals is needed; this type of culture is known as "**fed-batch culture**."
- Technically, there are not too many differences between batch and fed-batch culture.
- The fed-batch medium composition is similar to that of batch culture, commencing the culture process **with a lower viable cell density than the capacity of the bioreactor**.

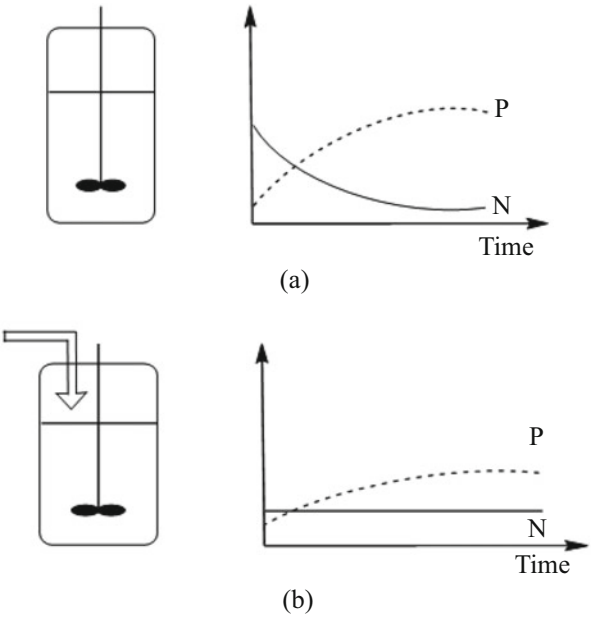

Fig. 13 Typical variations of cells (P) and nutrients (N) for (a) batch and (b) fed-batch fermentation modes

- As the cells continuously grow, there is progressive attainment of the stationary growth phase without any external manipulation including the administration of a new medium, etc., until the cells attain the stationary phase.
- "**Intermittent Harvest**: At this point, a portion of cells and product is harvested, following which the removed culture fluid is replenished with the fresh medium."
- For extended production duration, the process can be repeated several times.
- As the cells continue to grow exponentially, the nutrients in the medium undergo depletion. Owing to this, a concentrated feed medium (~ 10–15 times the dense basal medium) is refurbished either via continuous or at once mode. This restores the additional nutrients and facilitates an increment in cell concentration and the bio-product formation phase duration.
- Contrary to the discontinuous product harvesting, fresh medium is added as required by the cultured cells population, with no discard of culture broth.

NB: Generally FBBRs are used for fed-batch culture of mammalian cells.

- **In extended fed-batch culture**, cells are allowed to grow up and begin to feed on concentrated medium constituents. The viability continues to decline with the cell and product concentrations registering steady increments.
- A batch culture can attain a very high product and (microbial) cell concentration.

6.7.2 Fed-Batch Culture with Metabolic Shift

During the growth of cells either in batch or fed-batch mode, several metabolic by-products are accumulated in the culture. The most prominent metabolic products are ammonium and lactic acid. The other changes that take place in the cell culture medium are enhanced osmolarity and the accumulation of reactive oxygen species (ROS). The metabolic by-products and the molecular level changes may change the quality of the glycoproteins produced besides inhibiting cell growth. Therefore, the removal of these molecules from the cultured cells is highly essential.

Additionally, the culture is standardized in a manner that the cell metabolism begins with a low glucose concentration and then slowly advances to an efficient state, typically characterized by a dramatic reduction in lactic acid production.

"***Such a change in cell metabolism from the usual high lactate producing state to a much-reduced lactate generation is often referred to as metabolic shift***."

NB: The hybridoma technology used for the production of monoclonal antibody production is characterized by a very high cell density.

6.8 Continuous Flow or Perfusion Culture

- Perfusion allows for sustaining a cell culture bioreactor, wherein an equivalent volume of cell culture medium can be simultaneously added and removed, while the cells are retained in the bioreactor (Yang et al. 2007).
- This bioreactor needs a continuous supply of fresh medium and constant removal of **metabolic wastes**.
- To attain a higher cell density, commonly **perfusion** is used, which enhances the medium cell density to an extent higher than possible with a conventional bioreactor operating under batch or fed-batch conditions.
- The medium flow can be regulated using **a peristaltic pump** (**medium flow may create room for contamination**).
- The **microfiltration** procedure is used to harvest the secreted proteins for the cultured cells.
- For longer production times, the continuous flow or perfusion culture can be allowed.
- Due to the continuous addition of medium as well as continuous removal of toxic metabolites, there is no or very little fluctuation in the culture conditions including nutrient availability (Fig. 14).

  NB: Usually, the chemostat bioreactors are used for the continuous culturing of mammalian cells. A chemostat (implying unchanged chemical vicinity) is a bioreactor that works via regular supplementation of fresh medium and at the same time, the liquid having leftover nutrients, metabolic end-products, and cells is removed at the same rate. This ensures keeping the volume constant (Fig. 15).

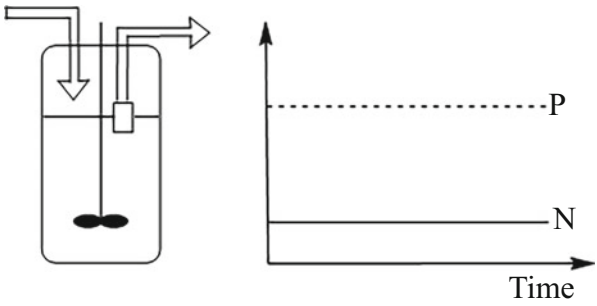

Fig. 14 Cell mass (P) and nutrient (N) variations for perfusion culture

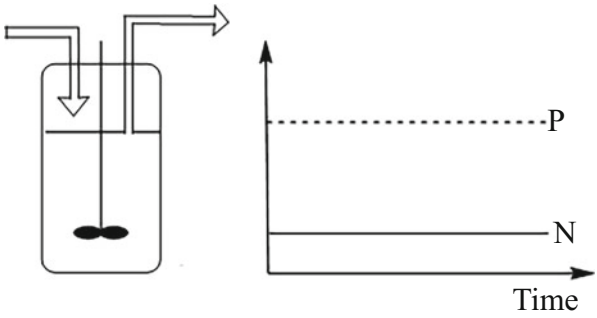

Fig. 15 Typical nutrition (N) and cell mass (P) variations for a Chemostat

6.8.1 Use of Perfusion to Improve the Mammalian Cell Culture

- Accomplishing a high Viable Cell Density (VCD) along with biomass productivity is important to improve the product quality. This also ensures upright product quality in multiple manners, which overall plays a big role in making the operation economically sustainable.

  Certain scenarios of attaining a cell culture perfusion are suggested by the following:

6.8.2 Continuous, Simultaneous Production, and Harvest

- In this case, the culture maintains a high cell density, and the product(s) is collected continuously for 30–90 days.
- From a relatively small bioreactor, consistent and efficient product quality could be accomplished.

6.8.3 High-Density Seed Banks

- Culturing via perfusion enables suspension mode growth of cells, wherein larger culture media volume could be preserved, allowing a rapid cell expansion start-up.
- Perfusion culture enables a collection of cultured cells in the log phase with a needed VCD alongside high quality.

6.8.4 Concentrated Fed-Batch Mode

- This mode of cell culturing makes use of an ultra-filter to preserve the generated bio-product in the bioreactor with the waste materials being perfused from the cell suspension.
- Typically of short duration, these cultures operate like those of fed-batch mode and could easily accomplish 5- to 10-fold greater cell densities and product quantities compared to the generally employed fed-batch mode.
- Owing to a higher yield of generated bio-products, these culturing modes of bioreactors could generate substantial savings from their operation in the production stage.
- The individual unit operations could be assessed to compute the savings incurred on raw material and processing expenditure in course of perfusion.

6.8.5 Advantages of Perfusion Culture

The following are the merits of perfusion culture:

- The instrument size may be small and flexible.
- Fast start-up in the process development.
- The culture environment may be better controlled.
- A continuous supply of nutrients.
- Longer, steady-state operation.
- Achieving high cell density and productivity.
- Better quality of the product.
- Better economic achievements.

6.8.6 Disadvantages of Perfusion Technology

The following are the demerits of perfusion culture:

- Potential licensing/regulatory issues.
- Comparatively high level of analytical costs.
- There are chances of contamination risk.
- Long duration validation time.
- Chances of equipment failure.

6.9 Cell Support System for Large-Scale Culture of Mammalian Cells

6.9.1 Suspension Culture Versus Adherent Culture

- For mammalian cell culture, various systems have been developed over the past several years.
- Several instruments are available for large-scale mammalian cell culture.
- Since most mammalian cells grow in an anchorage-dependent manner, these devices are manufactured to ensure the basal layer attachment of mammalian

cells while growing inside the containers. This type of culture is called **anchorage dependent culture**.

- For the successful and economic production of anchorage-dependent cells, the surface attachment requirement necessitates a large surface-to-volume ratio in the culture unit.
- However, some cells (e.g., white blood cells) are **anchorage-independent** and therefore do not need any supporting material for their culture. These cells are cultured **in large numbers as suspension**.
- Additionally, it is observed that suspension-cultured cells normally grow in large densities compared to anchorage-dependent cells, an economically benefitting prospect for production. Therefore, even the anchorage-dependent cells such as Chinese Hamster Ovary (CHO) cells which naturally grow as anchorage-dependent cells, are adjusted and adapted to suspension culture.

The scale-up of mammalian cell culture can be accomplished in the following two regimes:

1. Scale-up of anchorage-dependent cell culture
2. Scale-up of suspension cell culture

Here is a brief discussion about the scale-up of anchorage-dependent and suspension cultures.

1. **Scale-Up of Anchorage-Dependent Cell Culture**

For anchorage-dependent mammalian cell culture, the overleaf systems or working configurations could be very much useful.

Microcarrier System

- In the microcarrier cell culture system (**discovered by** Van Wezel 1967), cells are seeded and allowed to grow on microbeads which are subsequently kept suspended by stirring, as a suspension culture (Van Wezel 1967).
- The natural or synthetic molecules that are used as microcarrier supports include **denatured collagen, dextran, cellulose, gelatin, polystyrene**, and **Sephadex**.
- The static or packed beads are used to hold the carriers and the nutrient fluids are pumped through them.
- To prevent the packed bed from shifting, the **spheres of 3 or 5 mm** in diameter are packed quite tightly. However, it still allows the medium to flow through but without developing any shear stress.
- While the cells grow in a suspension with gentle agitation, these small spherical beads generally act as supports for cell growth.
- This system provides high cell yields from a small culture system, starting from 10^5 cells per ml this culture can provide 10^6 cells per ml.
- Therefore, it is an attractive procedure for the production of cells and various cellular products (Nilsson et al. 1986; Merten 2015).

Allowing the scale-up of anchorage-dependent cells in suspension mode can improve the followings:

Better control of culture parameters
Increased product yield
Reduced serum requirement
Lower contamination risk

Advantages of Microcarrier system

- Easier medium change and cell washing.
- It is easy to perfuse an immobilized monolayer of cells.
- The cell product formation (pharmaceutically important compounds, *for example*, interferon, Abs) is much higher.
- Repeated use of same setup and apparatus with different media and cells.

Disadvantages of Microcarrier system

- Tedious and costly.
- Requires more space.
- Provides more effective monitoring of cell growth.
- Difficult to measure and monitor the control parameters (e.g., O_2, pH, CO_2), etc.
- For the scale-up of monolayer cultures, a wide range of tissue cultures and systems have been developed. A selected few of them are briefly described.
- Of the other mammalian cell supporting systems, **microsphere induced cell aggregates and agarose microencapsulation** are the prominent ones.
- Microspheres were used to induce aggregate formation for CHO cells that grew either as aggregates or in suspension along with Vero and ST cells which exhibited a strictly anchorage-dependent growth. Unlike the cultivation on rather frequently used microcarriers or in the tissue culture flasks, the Vero and ST cells generally do not spread out while being attached to microspheres. The growth manner follows a rather spherical morphology and ultimately a rigid and dense cell mass. Irrespective of remaining growth conditions, cells mature as aggregation before attaining a high cell concentration. A pressing compulsion with the use of conventional microcarrier aided cell culture for attaining a similar cell concentration is the necessity of a huge quantity of microcarrier.
- For several diversified utilities of biotechnological and biomedical domains, viz. xenotransplantation, upkeeping a stem cell phenotype, and bioprinting of 3D scaffold for tissue engineering and regenerative medicine, a new assay involving the encapsulation of viable mammalian cells in a semi-permeable hydrogel matrix texture. Microencapsulation methodology typically comprises immobilizing the cultured cells within a polymeric semi-permeable environment which allows a two-way transport like inflow of oxygen, nutrients, growth factors needed for cell metabolism and the outer dissemination of discarded materials along with therapeutic proteins. The semi-permeability performs a critical function herein, forbidding the immune cells and Abs from damaging the encapsulated cells, presuming them as invaders.

6.9.2 Typical Scale-Up Procedure in a Mammalian Cell Culture Bioreactor

Start-Up Volume
5 to 100 ml in T flasks, shaker flasks, and roller bottles.

Intermediate Scale-Up
1 to 5 l in small, highly controlled bioreactors.

Large Scale-Up
20 to 10,000 l in large reactors.

6.9.3 Basic Steps of Scaling Up to 100 l Culture

STEP 1: Thaw **1 ml** mammalian cells vial.
STEP 2: Add to **75 ml** cell culture medium in a **500** ml roller bottle.
Cells grow to the appropriate cell number (~$1 \bullet 10^6$) within (1 to 3) days.
STEP 3: Put into **4 × 500** ml roller bottles.
Grow for (1 to 2) days.
STEP 4: All are pulled into a **10 L** bioreactor.
Grow them for 24 h.
STEP 5: After 24 h, divide them into 3, **10 L** bioreactors.
Grow for 24 h.
STEP 6: Put them into a **100 L** bioreactor and grow for (1 to 2) days.
STEP 7: If necessary grow further up to **10,000 L**.

NB: ***Culture time/days of cell culture may be changed depending on the type of cells being used, the type of cell culture medium with additives, and finally confluence of the culture containers. Nowadays bioreactors are used to produce various products including vaccines where as large as up to 10,000 l of culture medium is needed***.

6.9.4 Scale-Up of Suspension Cell Culture

- Scale-up of the suspension or anchorage-independent culture is easier than anchorage-dependent/adherent cell culture, and therefore, suspension culture is the preferred choice of scale-up mammalian cell culture.
- The cultured mammalian cells perform better as and when adapted from anchorage-dependent to suspension culture mode.
- At the initial stage of culture, the shake flask or spinner flasks are used with varying extents of agitation. The cultures are then transferred to bioreactors for large-scale culture with the typical volume being **100–10,000 l**.
- Anchorage-independent cultures are very adaptable; **it is possible to persuade suspension mode growth of many anchorage-dependent cell lines**.
- Generating such cell lines from an anchorage-dependent origin can be accomplished either via **selection or adaptation**.
- The selection procedure relies on the persistence of loosely attached strains within the population.

- Hematopoietic cells (blood cells) and hybridoma cells grow in suspension either as single cells or as clumps.
- Scaling up of these cells could be accomplished using stirred tank reactors or airlift bioreactors.
- Stirrers can be externally or internally driven. It is essential to maintain the pH and oxygen supply in both systems. A typical culture of **2×10^6 cells** can be scavenged off the oxygen within an hour.
- For supply of oxygen surface aeration, sparging membrane perfusion and membrane diffusion are done.
- The pH is maintained using a CO_2-bicarbonate system (Leist et al. 1990; Varley and Birch 1999).

  NB: The major limitation of scale-up in suspension culture is inadequate mixing and gas exchange.

  For small cultures, stirring the medium is easy, but the problem occurs with large cultures. The design of the bioreactor should facilitate maximum liquid movement with minimum shear damage to the cells.

6.9.5 Static Suspension Culture

- The culture of static suspension culture and the anchorage-dependent culture follow the same procedure.
- No agitation or mixing is involved in growing static suspension culture.
- One of the best examples of the cell line that grows well in static suspension culture is lymphocyte cell line mouse L1210.
- In general, it is difficult to scale up the cultured cells in static suspension mode, and therefore, it is not a preferred method of culturing.

6.10 Usefulness of a Bioreactor

A mammalian cell culture bioreactor has the following unique specifications concerning culturing specializations:

- Production of monoclonal antibodies (MAbs).
- Production of manifold vaccines.
- Production of various recombinant proteins.
- Production of potential enzymes.
- Aids in early cell screening and medium selection.
- Helps in cell line optimization.
- Disposable bioreactors utilize a disposable sterilized cell chamber to maintain the cell culture. This cell chamber minimizes the cross-contamination risk as it is used only for one growth operation. The use of disposable bioreactors decreases the extent of validation, cleaning, sterilization, and maintenance needed per bioreactor run. For this reason, disposable bioreactor runs can be scheduled closer, paving way for enhanced plant production.
- Several new bioreactors have been developed. These bioreactors include structural scaffolds that are adapted to grow as specialized cells and tissues to create

organ-like structures. Examples of organ-like structures equivalent to bioreactors are heart tissues, skeletal muscle tissues, ligaments, cancer tissues, and others. Currently, intensive research works are going on to enhance the production of these specialized bioreactors.

7 Conclusions

Various provisions are presently available for large-scale mammalian cell culture, optimized to cater to the need of various industrial purposes. For small to medium scale-up of the mammalian cell culture, the culture systems primed are spinner flasks, cell factories, roller bottle systems, hollow fiber systems, and small and medium-sized bioreactors. However, by increasing the number of above instruments such as spinner flasks, cell factories, roller bottle systems, and hollow fiber systems, the mammalian cell culture volume can be enhanced. Similarly, nowadays bioreactors are available with several thousand liters culturing capacity alongside robotically controlled monitoring of every culture step. This essentially reduces the contamination chances arising from human interception. Regarding culture procedure, suspension cultures are the most suited methods although most mammalian cells are adherent in nature. The inherent purpose of suspension culture is to get the cellular bio-products in high densities compared to an adherent culture, which essentially increases the number of specific products per liter of utilized culture volume. For this, adherent cells such as Chinese Hamster Ovary (CHO) cells require adjustment and adaptation from adherent to suspension mode. For harvesting, the specific products, batch, fed-batch, or continuous culture could be used. Nowadays, several advanced bioreactors have been developed by various research groups, typically used for growing specialized tissues/cells on a structural scaffold, in an attempt to recreate organ-like tissue structures in vitro. These large-scale mammalian cell culture provisions particularly various bioreactors have essentially become the inseparable domains of the noted biotechnological and pharmaceutical industrial revolution.

8 Cross-References

► Mammalian Cell Culture in Three Dimensions: Basic Guidelines
► Organ, Histotypic and Organotypic Culture, and Tissue Engineering

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Experimental Mammalian Cell Culture-Based Assays

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https://doi.org/10.1007/978-981-19-1731-8_16-2

Abstract

Mammalian cell culture-based assays offer multiple vital clues to understanding several distinct physiological and pathophysiological events related to cells. The key events in this regard comprise the effects of various drugs and toxins on cell survival, cellular metabolism and proliferation, cell adhesion and migration, programmed and nonprogrammed cell death (apoptosis/necrosis), generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and so on. This chapter briefly describes the fundamentals and importance of above-mentioned cellular events and protocols to track cellular events such as cell proliferation along with the pros and cons of every assay. All these assays have an implicit association with mammalian cell proliferation, survival, adhesion, migration, apoptosis, and measurement of ROS and RNS generation levels. A discussion of these issues will enhance the reader's knowledge regarding mammalian cellular physiology as well as pathophysiology under in vitro (cell culture) conditions.

Keywords

Cell viability/cytotoxicity assay · Proliferation assay · Adhesion/Attachment assay · Migration assay · Apoptosis assay · ROS measurement · NO measurement

1 Introduction

This chapter describes the various assays related to mammalian cell culture, particularly cell viability and toxicity, cell proliferation (increasing number of cells), survival and apoptosis, cell attachment or adhesion, and cell migration. Additionally, various procedures and methods for quantifying reactive oxygen species (**ROS**) and reactive nitrogen species (**RNS**) are described. Cell viability determines the number of viable cells in an experimental cell culture sample. The assays described here are based on membrane integrity such as dye exclusion assay (e.g., trypan blue exclusion assay), dye uptake assay (labeled chromium uptake assay), and enzyme release assay (e.g., lactate dehydrogenase release assay). Other assays described here include those based on luminescence test (level of ATP determination), clonogenic assay: MTT assay (ascertaining cell proliferation and toxicity), and an assay based on radioisotope incorporation: Thymidine incorporation assay (thymine is one of the four constituent nucleotides of DNA. During DNA synthesis, thymine/radiolabeled thymine is incorporated into the DNA and thereby correlated with the new DNA synthesis and cell proliferation). Other important assays briefly discussed here are as follows: assay based on colorimetric quantification, an assay based on cellular respiration and metabolism. Programmed death of the cells, called apoptosis, is one of the important physiological events to study cancer biology, immunology, cell biology, and other aspects. The different apoptosis determination assays

described here include Annexin V binding of cell surface phosphatidylserine, DNA condensation and fragmentation (**TUNEL**) assays, caspase activation and detection assays, and cytochrome C release. Except for some leukocytes (e.g., B or T cells), most mammalian cells are adherent in nature. Adherent or attachment properties are highly essential for mammalian cell growth and proliferation. Also, cancer cells needed differential expression of adhesion molecules for their invasion, migration (metastasis), and growth over to a new place. Hence, cell adhesion and migration assays are essential aspects of mammalian cancer biology. Besides adhesion assay, this chapter describes two different migration assays, namely, in vitro scratch assay and **Boyden chamber assay**. Finally, this chapter describes the various methods to measure ROS and RNS. While ROS and nitric oxide (NO) at the physiological level may act as secondary messengers and fulfill various physiological functions at the supraphysiological level, both ROS and RNS complicate various diseased conditions. Therefore, the measurement of ROS and RNS comprises an essential requirement of mammalian cell biology. All these assays related to mammalian cell proliferation, survival, adhesion, migration, apoptosis, and measurement of ROS and RNS levels will enhance the reader's knowledge regarding mammalian cellular physiology as well as pathophysiology under in vitro cell culture conditions.

Here is the chronological briefing of the various assays:

2 Cell Viability/Cytotoxicity and Cell Proliferation Assays

- **Cell viability** can be defined as the number of healthy cells in a sample. It determines the population of cells (regardless of cell-cycle phase) that are living, out of a total cell sample. In the mammalian cell culture experiments, cells are isolated from mammalian tissues/organs and then subjected to culture in a CO_2 incubator, in an in vitro environment. The viability of the cells represents the capability of their existence, survival, and division (proliferation).
- Studies on **cell cytotoxicity** broadly involve the metabolic alterations of the cells, including cell death, occurring as a result of toxic effects of various compounds, such as experimental drugs or toxins. For instance, in the case of anticancer drugs, one may look for cell death, while for cosmetics, the metabolic alterations and allergic responses may be more important (Mosmann 1983; Pegg 1989; Marshall et al. 1995; Berridge et al. 1996; Tominaga et al. 1999; Riss et al. 2013).

Cell viability/cytotoxicity and cell proliferation assays have the following importance

- To determine the undamaged, live, and physiologically active cells.
- To assess the success of cell culture (cell proliferation/death) besides optimized growth conditions and culture medium, doing transfection experiments.
- To determine the number of cells undergoing programmed cell death (apoptosis).
- To determine the success of cryopreservation techniques.
- To determine the success of transplant acceptance or rejection.

- To determine the in vitro toxicity of various drugs, chemicals, cosmetics, and other molecules on various experimental cells including normal or cancer cells.
- To diagnose a diseased condition.
- To diagnose male infertility.
- To ascertain deformability, osmotic fragility, hemolysis, ATP expression, hemoglobin content of the red blood cells, and so on.

2.1 Types of Cell Viability/Toxicity and Cell Proliferation Assays

There are several laboratory-based assays known to ascertain cell viability and cytotoxicity.

The different cell viability and cytotoxicity assays are based on the following:

1. Assays Based on Membrane Integrity
   Dye Exclusion Assay: **Trypan Blue Exclusion Assay**
   Dye Uptake Assay: **Labeled Chromium Uptake Assay**
   Enzyme Release Assays: **Lactate Dehydrogenase Release Assay**
2. Assay Based on Luminescence Test: **Determination of The ATP Level**
3. Clonogenic Assay: **MTT Assay**
4. Assay Based on Radioisotope Incorporation: **Thymidine Incorporation Assay**
5. Assay Based on Colorimetric Determination
6. Assay Based on Cellular Respiration
7. Metabolic Assay

Here is a brief discussion of the above assays:

2.1.1 Assays Based on Membrane Integrity

- Checking the membrane integrity is the most common cell viability measurement technique.
- During mammalian cell isolation and culture, several factors may affect the membrane integrity of the cells. The most prominent factors may be proteolytic enzyme-treated cell disaggregation during slow freezing and rapid thawing of cells.
- The simplest assay to check the membrane integrity of cultured mammalian cells is to check the uptake of various dyes by these cells. While live cells with healthy biological membranes do not uptake any dye (impermeable to dyes such as trypan blue), the **injured or membrane damaged cells** do take up various dyes such as **naphthalene black, trypan blue, and erythrosine** and therefore stain the cells.
- On the other hand, **neutral red** can be transported into the live cells by the active pump mechanism and dead cells cannot be stained by this dye.
- Similarly, the **fluorescein diacetate stain** is an esterase substrate that reacts with live cells' **esterase** enzyme and hydrolyzes it into a **green fluorescein compound**.

- The release of **labeled chromium (^{51}Cr), enzyme**, and the use of fluorescent probes are the other assays for determining membrane integrity.
- Cell viability measurements, based on membrane integrity, are spontaneous and can be detected within a few hours.
- The leaked membrane-containing cells can also be stained using propidium iodide, by determining the amount of lactate dehydrogenase (LDH), a stable enzyme common to all cells and could be readily detected when cell membranes are no longer intact.
- The ultimate survival of mammalian cells cannot be predicted by the above assays.

2.1.2 Dye Exclusion Assay

The live mammalian cells with the normal plasma membrane (without any injury or damage) oppose the staining by certain dyes such **as erythrosine B, eosin Y, nigrosine green, trypan blue,** and **naphthalene black. However, in membrane-damaged cells, these dyes can enter the cells and stain the cytoplasm**.

- The technique consists of mixing the cells in suspension with the dye before examining them under the microscope.
- The stained cells and the total number of cells are counted.
- The percentage of unstained cells ascertains the number of viable cells.
- Dye exclusion assay is convenient but is more suitable for suspension cultures than monolayers.
- This is because as the dead cells detach from the monolayers they are lost from the assay. The major limitation of this assay is the inadequacy of reproductively dead cells for not taking up the dye, owing to their mistaken consideration as being viable.

In the following paragraphs, the trypan blue exclusion method is described.

2.1.3 Cell Viability Testing with Trypan Blue Exclusion Method

- In mammalian physiology, the live-cell membrane maintains the structural integrity of the lipid bilayer and does not allow dyes (like Trypan blue) to enter the cytoplasm.
- However, damaged proapoptotic or dead cells lose their membrane integrity and bleb. Through this damaged membrane, dye like trypan blue can easily enter the cell cytoplasm and stain it into a blue color, facilitating easier observation under a light microscope.
- Thus, blue-stained cells are damaged/proapoptotic or dead cells while their opposite, nonstained cells are live cells. Trypan blue is also used to eliminate false positives during cell counting by flow cytometry (Strober 2015).

NB: *Trypan blue is a carcinogenic agent owing to which, suitable protection is needed while working with it as also that of wearing hand gloves*.

Equipment and Materials for Trypan Blue Exclusion Assay

Light microscope/inverted microscope.
Hemocytometer and coverslip.
Cryovials.
Pipettes and tips.
Trypan blue and mammalian cells.

Method of Trypan Blue Exclusion Assay

- Make a cell suspension and take **50 µl** of it in a microfuge.
- Add equal parts of 0.4% trypan blue to the cell suspension to obtain one or two dilutions (example: **50 µl cell suspension + 50 µl trypan blue = 100 µl**) followed by thorough mixing via pipetting up and down. Trypan blue is soluble in **0.1 mg·ml^{-1} water**.
- Keep the mixture for 2 to 3 mi at room temperature.
- **NB**: Cells should not be counted more than 5 min after mixing with trypan blue. This may be related to more number of cells that died during the counting.
- For cell counting, first put the cover slip at the proper place on the hemocytometer.
- Draw 10 to 20 µl of cell suspension with help of a micropipette.
- Put the pipette tip at the notch and fill one side of the hemocytometer counter.
- Take the hemocytometer and place it on the stage of a light microscope or inverted microscope.
- Now focus on the cells.
- As one observed through the microscope, multiple squares are located on the hemocytometer.
- Each large square contains 16 small squares. Count the cells that are on the border lines on two slides only.
- Count the blue cells as damaged membrane-containing cells (may be dead or pro-apoptotic cells/nonviable cells) [Refer to Fig. 1 for a better idea].
- Separately, count the transparent cells as live cells.

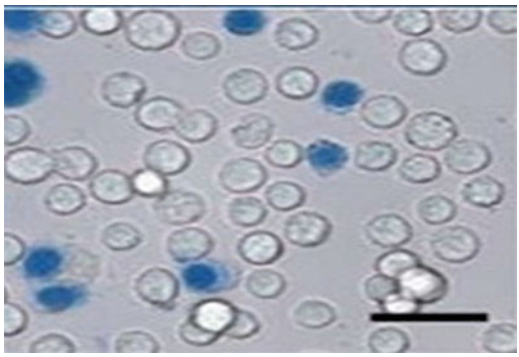


Fig. 1 Pictorial view of cell population in the Trypan Blue Exclusion Assay. The blue-colored cells are considered either proapoptotic or apoptotic

- Calculate the percentage of viable cells via dividing the number of viable cells by the number of total cells and multiplying by 100 for obtaining % viable cells, using the following equation,

$$\left[1 - \left(\frac{\text{Number of blue cells}}{\text{Number of total cells}}\right)\right] \text{X } 100$$

NB*: Instead of using trypan blue, some may prefer to use Erythrosine B*.

- It is recommended that Erythrosine B is not a potential carcinogen like Trypan blue.
- Other benefits of using Erythrosine B include:
- It does not require any incubation with the cells.
- It does not bind to serum protein.
- It shows a much cleaner background than Trypan blue.

2.1.4 Dye Uptake Assay

- This assay applies only to the live cells.
- Diacetyl fluorescein acts as an ester which would be hydrolyzed by the esterase enzyme of the live cells.
- This leads to the generation of green fluorescent light.
- Now, the viable cells are identified by observing the emitted green fluorescent light.

2.1.5 Labeled Chromium Uptake Assay

- The basic amino acids of the intracellular proteins bind to the **^{51}Cr**.
- The labeled proteins leak out when the membrane is damaged. This leaking extent is generally proportional to the damage extent.
- The labeled ^{51}Cr uptake method is used in immunological studies to determine the cytotoxic activity of T-lymphocytes against the target cells.

Here is a very brief summary of the chromium release assay:

- Grow the target T cells in a 96-well plate.
- Incubate these cells with ^{51}Cr.
- Reincubate the target cells with effector cells (your T cells; E).
- If the effector cells kill the target cells, they will release ^{51}Cr, which could be detected using a γ-counter.

NB: *The type of targets and effectors one uses, is entirely dependent on applications*.

2.1.6 Enzyme Release Assays

- The injured or damaged mammalian cells release various enzymes that can be estimated.
- The most prominent of these enzymes is **Lactate dehydrogenase (LDH), an oxidoreductase**.
- **Under anaerobic and stressful/cell damaged conditions, pyruvic acid, a product of glycolysis in the cell cytoplasm, is converted to lactic acid by LDH**.
- The LDH is released into the bloodstream after tissue damage or the hemolysis of red blood cells (Burd and Usategui-Gomez 1973; Korzeniewski and Callewaert 1983; Decker and Lohmann-Matthes 1988).

Equipment and Materials for Lactate Dehydrogenase Release Assay

- Flat bottom, 96-well microtiter plates.
- A plate reader.
- Centrifuge with plate adaptors.
- Multichannel pipettes and repeat pipettor.

Tris Buffer

Dissolve 24.2 gm Tris base in 1-l milli-Q water. Make a 200 mM solution **at pH 8.0**.
Adjust pH to 8.0 using hydrochloric acid.
Sterilize through autoclaving.

2X LDH Assay Buffer

- Take 480 ml, 200 mM Tris-HCl at pH 8.0. To this, add and dissolve 3.2 gm lactic acid, 575 mg nicotinamide adenine dinucleotide (**NAD**), 223 mg 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (**INT**), and 57 mg N-methylphenazinium methyl sulfate (**PMS**).
- Make small aliquots of the 2x buffer and store at −20 °C.
- Repeated freezing and thawing are not recommended.
- Finally, store the buffer in an aliquot.

10X Lysis Buffer

9% Triton X-100.
Dissolve 9 ml Triton X-100 in 91 ml milli-Q water (deionized water).

Stop Solution

1 M acetic acid and (1–5)% SDS

A strong base (such as 1 N NaOH) or strong acid (such as 1 N hydrochloric acid (HCl)) can be used to stop the reaction.

Method to Measure Lactate Dehydrogenase Release

- Take one 96-well flat bottom microtiter plate.
- Add 100 μl cell suspension in cell culture medium [**(1 to 5) X 10^4 cells**] per well.
- **NB**: Different cells contain varying LDH extents. Therefore, the optimum cell number for each assay must be standardized.

- Incubate the cells at 37 °C, with 95% moisture and 5% CO_2.
- Add 10 µl, 10X lysis buffer to the LDH release wells and the volume correction control wells.
- Return the plate to the same CO_2 incubator and incubate for 45 min.
- Centrifuge the microtiter plate at (1500 to 2000) rpm for 5 min.
- The centrifugation step minimizes the cell culture supernatant contamination with cell materials.
- With a multichannel pipette, collect 50 µl culture supernatant without taking any cell from each well and transfer it to a new microtiter plate.
- In each well, add 50 µl reconstituted 2x LDH assay buffer to the supernatant (**50 µl 2x LDH buffer + 50 µl culture supernatant**).
- **NB**: Depending upon the sensitivity, a smaller culture medium volume may be used. The sensitivity of the assay depends upon several factors including the number of cells used, the amount of LDH expressed in a given cell type, and the reagents used.
- Gently shake for 30 s to mix the contents.
- The microtiter plate content is light-sensitive. So, cover it with aluminum foil and incubate at room temperature (22 to 25 °C) for 10 to 30 min.
- Depending on the color development, the reaction time can be decreased or increased.
- Periodically monitor the color conversion.
- The color should be stopped (add **50 µl stop solution** and mix for 30 s) before the sample reaches a level corresponding to that of maximum LDH release wells.
- Measure the absorbance at 490 to 520 nm within 1 h after administration of the stop solution.
- However, the administration of the stop solution is not required if the rate of conversion is too slow.
- **NB**: If the immediate measurement is not possible, store the cell-free culture supernatant in a refrigerator (2 to 8) ^{o}C for a couple of days, ensuring no significant loss of activity.
- While the culture supernatant is utilized for the LDH assay, the leftover cell pellet may be utilized by other cell viability assays such as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium).

Data Analysis

- The values from untreated and test walls are subtracted from that of the medium alone (control cells).
- The readings from the untreated and test wells are subtracted from those of the medium alone (control wells).
- The obtained values are the corrected reading corresponding to the untreated or test wells.
- The value of the volume correction control wells now needs to be subtracted from the maximum LDH release wells.
- This is the corrected maximum release reading.

- The cytotoxicity computation for the test can be made using the following equation:

$$\%\text{Cytotoxicity} = \left(\frac{\text{Corrected reading from test well-Corrected reading from untreated well}}{\text{Corrected maximum LDH release control-Corrected reading from untreated well}}\right) \times 100$$

NB: The experiment may include a positive control of the LDH standard.

For successful completion of the LDH assay, the following points are important:

- The assay is performed at room temperature (22 to 25 °C). Therefore, every chemical must be warmed at room temperature before commencement.
- The LDH assay buffer constituents are light sensitive. So, these should be protected from light using various materials such as aluminum foil.
- The culture medium should not contain dithiothreitol (DTT), β-mercaptoethanol, and ascorbic acid since these reducing agents nonenzymatically reduced the INT.
- In this assay, the used PMS is recognized as an electron carrier for the second step of the LDH reaction. Therefore, Mendola blue or diaphorase can also be used as an electron carrier.
- Phenol red, the pH indicator of the cell culture medium may interfere with the absorbance. For better results, a phenol red-free medium should be used.
- Pyruvate, the common mammalian cell culture medium constituent may not be added to the herein, since it could be converted to lactic acid by LDH.
- The fetal bovine serum (**FBS**) or fetal calf serum (**FCS**) that is used as one of the major nutrient constituents of most of the cell culture media, may contain various amounts of LDH. Therefore, the serum constituent in the medium would be as less as possible to moderate the background LDH content.

2.2 Assay Based on Luminescence Test

2.2.1 Measurement of Adenosine Triphosphate by Luciferase Assay

- **Adenosine Triphosphate (ATP)** is present in all organic cells and is the universal unit of energy used by all living cells.
- Metabolically active cells produce **ATP, (the energy currency of the cells)**, an indicator of their corresponding viability.
- Hence, ATP can be used to determine a healthy physiological status and the cytotoxic effects of the mammalian cells.
- In fireflies, the luciferase enzyme converts a compound called luciferin into oxyluciferin, producing light or "**luminescence**" as a result. This reaction **requires** energy derived from ATP to proceed, so researchers have exploited the luciferase-luciferin interaction to gauge ATP levels in cells (Fig. 2).
- ATP is quantified by measuring the light produced via its reaction with the naturally occurring firefly enzyme, luciferase using a **luminometer**.

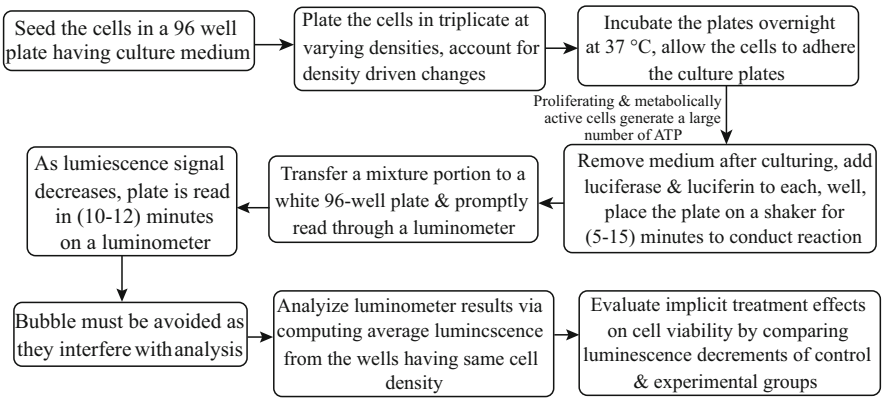


Fig. 2 Chronological sequence of Luciferase Assay steps for determination of Adenosine triphosphate

- The amount of light produced is directly proportional to the ATP concentration of a tested sample.
- The luminescence-based test measured the ATP contents of the cells, an indication of cell viability.
- Good sensitivity of this test is reported for the cells within the **(20 to 25) X 10^7** cells·ml^{-1} range (Turman and Mathews 1996).

2.2.2 Equipment and Materials for Luciferase Assay

- Flat bottom, 96-well microtiter plate.
- Microplate reader.
- Centrifuge with plate adaptors.
- Multichannel pipettes and repeat pipettor.
- Luminometer.
- Luciferase and Luciferin.

2.2.3 Method to Measure Adenosine Triphosphate in the Cells

Take a 96-well cell culture plate and seed cells at various densities to account for density-dependent variations.

Incubate the cells in a **37 °C incubator, 5% CO_2,** and **95% moisture** environment. Allow the cells to adhere to the culture vessels.

- The metabolically active and proliferating cells produce a large number of ATP.
- Complete the duration of the cell culture and remove the medium.
- Add luciferase and luciferin to each well.
- Put the plate on a shaker for 5 to 15 min to mix the luciferase and luciferin with the cells.
- Transfer a portion of the mixture into a white 96-well plate and read through a luminometer immediately.

- The plate should be read within 10 to 20 min on a luminometer since the luminal signal decreases over time.
- The bubbles can interfere with the results.
- Researchers can evaluate the comparative luminescence data from both healthy control samples and treated cells on viability and metabolism of the cells inferred by the decreasing luminescence in the experimental group.

2.2.4 Clonogenic Assay

In the clonogenic assay, the survival of the cells is measured by the plating efficiency (i.e., the percentage of cells seeded at a subculture that gives rise to colonies) (Buch et al. 2012). The plating efficiency determines the proliferative capacity for several cell generations.

Following are the steps involved in the clonogenic assay:

- Grow and treat the cells with the desired experimental agents for a fixed duration.
- Trypsinized the cells.
- Now seed the cells at a very low density so that each cell gets the chance to form individual colonies.
- To form the colonies, the cells should be incubated for 1 to 3 weeks.
- Once colonies are formed, they are fixed and stained with crystal violet.
- The visible cells are now counted.

Factors Affecting Clonogenic Assay

- The concentration of the toxic agent.
- Exposure duration of the toxic agent.
- The density of the cells during exposure.
- The density of the cells during cloning.
- The size of the colony.

MTT Assay

- MTT is a tetrazolium salt (dye) with IUPAC name 3,(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
- This is a type of colorimetric assay for measuring the activity of the enzyme (mainly via **mitochondrial dehydrogenase**).
- The live cells produced **NADH** and **NADPH**, which are also required for the enzymatic conversion of MTT.
- These enzymes **reduce** MTT and structurally similar dyes such as **MTS, XTT,** and **WST** to purple-colored **formazan**. Various reductase enzymes are active only in live cells, and therefore, this assay determines the live cells (Fig. 3).
- The assay measures cell viability and proliferation.
- Additionally, the effects of potential experimental **drugs, toxins, or other molecules** that affect cell viability and proliferation, can also be measured using this assay.

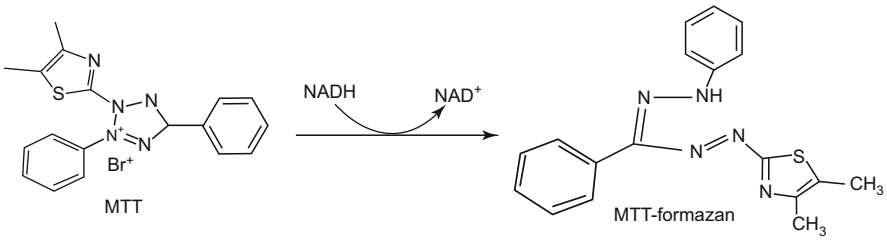


Fig. 3 Scheme for MTT to MTT-formazan conversion (the standard MTT assay reaction)

- A comparative analysis of the formazan produced by the treated and untreated cells would reveal the effectiveness of various experimental drugs on cultured mammalian cells (Berridge and Tan 1993; Riss et al. 2013).

Materials and Reagents Needed for MTT Assay

- Flat bottom, 96-well microtiter plate, and plate reader.
- Centrifuge with plate adaptors.
- Multichannel pipettes and repeat pipettor.
- Cell counter/Hemocytometer.
- MTT.
- DMSO or formazan solubilizing agent.

Method for MTT Assay

- Usually, (5000 to 10,000) cells are seeded into each well of flat bottomed, 96-well tissue culture plates. Cell numbers may go up to 100,000 as and when deemed by a specified protocol.
- Cells grow for (24 to 72) hours or according to the experimental need. However, cells must be in the logarithmic growth phase for conducting the MTT assay.
- For treatment, generally, 100 μl cell culture is taken with (5 to 10)% FCS and subjected to treatment with the optimized dose of the drug.
- Following drug treatment, 10 μl MTT solution is added to each well.
- Mix by tapping or shaking on an orbital shaker for 2 to 3 min.
- Place the plate in an incubator at 37 °C and keep it for 4 h. The incubation time may require standardization. MTT is a light-sensitive molecule. So, the plate needs to be covered with aluminum foil.
- Remove the medium containing MTT.
- To dissolve the formazan, add 200 μl DMSO to each well. Now mix it by pipetting up and down.
- Now measure the absorbance in an ELISA plate reader at 570 nm. However, any filter that absorbs 550 to 600 nm could also be used.
- For the analysis of the data, cell number versus absorbance needs to be plotted, which gives the varied cell proliferation extents.
- **NB**: The rate of cell proliferation is proportional to the rate of tetrazolium reduction.

2.2.5 Assay Based on Radioisotope Incorporation: The Thymidine Incorporation Assay

- [3H]-Thymidine is a labeled DNA precursor and [3H]-Uridine is a labeled RNA precursor.
- Incorporation of [3H]-Thymidine into DNA and [3H]-Uridine into RNA respectively signifies the active replication and transcription, directly ascertaining the cell proliferation.
- **Before a cell divides, its DNA precursors are incorporated and DNA is replicated**.
- Thus, as and when the cells undergo proliferation, the nucleotide precursors such as labeled thymidine would be incorporated into the cells for the DNA synthesis by the process of replication in the S phase of the cell cycle.
- The labeled thymidine-incorporated DNA can be isolated and measured using a scintillation counter (Friedman and Glaubiger 1982).

Equipment and Materials for Thymidine Incorporation Assay

- Flat bottom, 96-well microtiter plates.
- A microtiter plate reader.
- Centrifuge with plate adaptors.
- Multichannel pipettes and repeat pipettor.
- [3H]-Thymidine.
- Scintillation vials, fluid and counter.
- Membrane filter.
- Mammalian cells and cell lysis buffer.

Method to Measure Thymidine Incorporation

- Cell culture should be standardized into 96-well plates with 100 µl volume.
- Grow the cells up to (40 to 50)% confluency.
- To each well, add a 50 µl medium containing 0.075 µCi 3H-thymidine (specific activity 3167.2 TBq/mmol ≅ 85.6 Ci $mmol^{-1}$).
- Incubate for another (18 to 24) hours.
- Cells are harvested on a membrane filter having a specific pore size and lysed, leaving the DNA free.
- Now the lysed cell materials and DNA are passed through the membrane filter.
- While larger molecules such as DNA cannot pass through the membrane, the smaller ones can easily pass through if the size of the membrane filter pores is bigger than the lysed cell materials.
- The membrane is thereafter, transferred to plastic scintillation vials, 5 ml scintillation fluid is added and the counts per minute (CPM) are evaluated on a scintillation counter.
- The typical proliferation rate is proportional to the extent of radioactive molecules. This indicates that the greater the radioactive materials, the more the amount of DNA, which again is proportional to the number of cells.

Labeled Phosphate

- The cells are prelabeled with ^{32}**P**.
- In case of any accidental damage to cells, they release the labeled phosphate which can be measured.
- The efficacy of a drug can be evaluated using this approach.

2.2.6 Assay Based on Colorimetric Determination

In the case of the colorimetric assay, sophisticated microplate readers are used for reliable cell quantification.

The following are the chronological steps of a colorimetric assay:

- Protein content can be estimated using methylene blue, amido black, sulforhodamine, Folin-Ciocalteu reagent, etc.
- Various protein determination techniques are presently in use.
- The most widely utilized of them comprise Lowry, Bradford, and Bicinchoninic acid (**BCA**).
- DNA can be quantified via staining with fluorescence dyes, e.g., 2-diamidino-phenylindole (DAPI).
- Lysosomal and Golgi body activity can be ascertained using neutral red dye.
- Enzyme activity assays, e.g., hexosaminidase, and mitochondrial succinate dehydrogenase, are also used.

2.2.7 Assay Based on Cellular Respiration

Use **Warburg Manometer** and measure **oxygen utilization** or **carbon dioxide** production to examine the cell viability.

Metabolic Assays

- The measurement of cellular metabolic response is the basis of the metabolic assay.
- Grow the cells for 2 to 3 doubling times.
- Expose the cells to cytotoxic drugs.
- Measure the concentration of DNA, RNA, and proteins.
- Certain dehydrogenases can also be measured.

Limitations of Metabolic Assays

- The estimation of the total DNA/protein content may or may not be indicative of an enhanced cell population.
- The discrimination between proliferative assay and metabolic activity of the cells cannot be made using these assays.
- Some scientists, therefore, prefer to confirm the metabolic measurements via clonogenic survival assay.

3 Determination of Apoptosis

- Programmed cell death of a multicellular organism is called **apoptosis**.

Apoptosis is an entirely normal, natural physiological process with the following benefits:

- To remove or eliminate the aged or damaged and irreparable cells as a process of maintaining homeostasis by "**cell renewal system**." This will always maintain an adequate cell population in a mammalian body such as a human.
- To physiologically develop an embryo or fetus by removing the unwanted cell or tissues such as tissues in between the **fingers** of hands and legs of humans.
- To maintain a proper mammalian (e.g., human) body size.

The Nomenclature Committee on Cell Death (NCCD) has proposed that a cell should be regarded as "dead" when it exhibits at least the following three symptoms:

- Lost integrity of its plasma membrane.
- Complete disintegration including that of the nucleus.
- Its corpse (or its fragments) has been engulfed by a neighboring cell in vivo with no prevalence of an inflammatory reaction.

Other important characteristics of an apoptotic cell are as follows:

- Shrinkage of the cell and the nucleus.
- Loss of adhesion to the cell-culture containers as well as to the neighboring cells.
- Blebbing of the membrane by moving inner membrane materials to the outer membrane.
- Nuclear fragmentation, chromatin condensation, and mRNA decay.
- Formation of the apoptotic bodies (Elmore 2007).

NB: *Apoptosis can be induced by several factors including the withdrawal of the growth factors, loss of matrix attachment, activation with glucocorticoids, some viruses, free radicals, ionizing radiations,* etc.

- Besides apoptosis, **necrosis** is another mechanism of cell death.
- Necrosis is considered a **nonprogrammed cell death**.
- In general, necrosis occurs when the cells are exposed to toxic agents or extreme variations in physiological conditions due to various reasons including **hypothermia, hypoxia**, etc., leading to cell membrane damage.
- Under necrotic conditions, the homeostatic ability of the cells gets impaired, leading to an influx of water and extracellular ions within the cells.

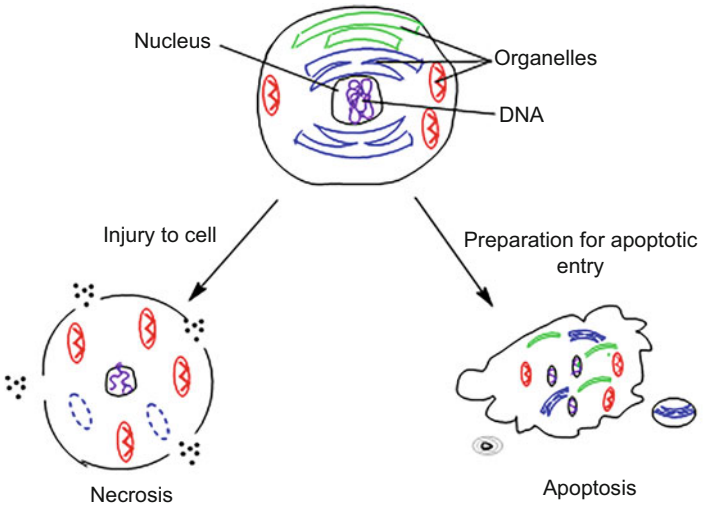


Fig. 4 Different fates of an injury-affected cell

- This further leads to **cell swelling, including swelling of the intracellular organelles such as mitochondria**.
- Finally, the necrotic cells would be lysed.
- The damaged/lysed cells and tissues, gradually get involved in the large-scale accumulation of **inflammatory molecules, followed by inflammatory reactions**.
- Figure 4 outlines the distinction between apoptosis and necrosis-mediated cell death.

3.1 Process of Apoptosis

- Once apoptosis is activated, it leads to physiological death of the cells.
- The signals for apoptosis are either originated from outside the cells called an **extrinsic pathway** or may originate from the intracellular organelles, namely **mitochondria**, and therefore called an **intrinsic pathway**.
- In the case of the extrinsic pathway, the molecules such as **TNFα** and Fas bind to their **cognate receptors** on the plasma membrane. The receptor–ligand interaction activates cell surface death receptors, which leads to the formation of the death-inducing signaling complex (**DISC**).
- On the other hand, in the case of the intrinsic pathway, the intracellular membrane proteins such as **cytochrome C** are released from the mitochondria into the cytoplasm, leading to the activation of the intrinsic pathway.

4 Determination of Apoptosis

Several methods are now used to determine the apoptosis of the cells.

Some of the prominent methods are as follows:

1. Annexin V Binding of Cell Surface Phosphatidylserine.
2. DNA Condensation and Fragmentation (**TUNEL**) Assays.
3. Caspase Activation and Detection Assays.
4. Cytochrome C Release Assays.

Here is a brief discussion of the various (above) apoptotic assays:

4.1 Annexin V Binding of Cell Surface Phosphatidylserine

- The changes in the early phases of apoptosis can be identified by the alteration of the cell surface plasma membrane.
- Careful observation revealed that **phosphatidylserine (PS), a membrane phospholipid flip-flopped (moved)** from the inner layer to the outer layer of the plasma membrane.
- The PS is well recognized as an **apoptotic marker protein**.
- The PS exhibits a strong affinity toward **Annexin V (**also known as **Annexin A5), a** $\mathbf{Ca^{2+}}$-dependent protein.
- While in the case of both apoptosis and necrosis, the PS flip-flopped from the inner membrane layer to the outer membrane, the necrotic cells membrane becomes damaged, leaky, and finally swells and gets lysed. The binding of Annexin V with the outer membrane PS is an indication of apoptosis.
- The Annexin V is conjugated with various dyes **(fluorochrome)** such as **propidium iodide, 7-amino-actinomycin D, and fluorochrome**.
- "The annexin V affinity assay measures the fraction of cells that are annexin V negative and propidium iodide negative; annexin V positive and propidium iodide negative; or annexin V positive and propidium iodide positive."

The assay requires mandatory attainment or absence of the following conditions:

The labeled Annexin V should not be stored in the deep freezer (-20 °C) as it could result in **dimerization** and loss of activity. Therefore, it can be refrigerated (2 to 8) °C.

- While the assay is recommended for conduction in the cell culture medium such as **DMEM** and **M199**, the **RPMI-1640** medium is not deemed suitable since it contains high phosphate and low Ca^{2+}.
- In absence of a medium, it is still wise to use **HEPES** or **Tris buffer**, but not **phosphate buffer**.

- For a detachment of the cells, **trypsinization** can be avoided since it affects the Annexin V binding of the cells. Instead of trypsinization, a **cell scraper** may be used.
- Cells should not be fixed using **paraformaldehyd**e before the addition of Annexin V since it may induce the PS expression by the live cells.
- Ca^{2+} at a concentration of **0.5 to 3 mM** is necessary for the binding of Annexin V with the PS.
- The concentration of Ca^{2+} should not be more than 5 mM since, at this concentration, the affinity of Annexin V with other phospholipid molecules such as phosphatidylcholine also increases.
- However, the affinity of the Annexin V with the PS would be lost at **<5.2 pH** or on prolonged incubation **above 42 °C**.
- The addition of Ca^{2+} chelating agents such as EDTA also results in loss of Annexin V binding with the PS.
- "Flow cytometry of the resulting cell suspension produces an annexin V versus propidium iodide dot plot with living cells (annexin V negative and propidium iodide negative), living cells with a compromised membrane due to scrapping (annexin V negative and propidium iodide positive), dying cells (annexin V positive and propidium iodide negative) and secondary necrotic cells (annexin V positive and propidium iodide positive)" (Bossy-Wetzel and Green 2000; Genderen et al. 2006).

4.1.1 Equipment and Materials for Annexin V Assay

Flow Cytometer.
Confocal Scanning-Laser Microscope.
Green Fluorescent Annexin V or Annexin A5.
Antibody to Fas.
Bovine Serum Albumin **(BSA)**.
Ionomycin.
Glycerol.
Paraformaldehyde.
Propidium Iodide.

4.1.2 Binding Buffer (10X)

250 mM HEPES plus NaOH, pH 7.4,
1.4 M NaCl
10 mM $CaCl_2$.

4.1.3 Binding Buffer (1X)

- 25 mM HEPES plus NaOH, pH 7.4,
- 140 mM NaCl
- 1 mM $CaCl_2$.

NB: Filter sterilizes the buffer and stores the binding buffer at 2 to 8 °C for up to 6 months.

4.1.4 The Fixation Buffer (4% PARAFORMALDEHYDE, 100 Ml)

- Add 4 g paraformaldehyde in 80 ml distilled water.
- Add a few drops of 5 M NaOH.
- Heat the solution to facilitate paraformaldehyde dissolution.
- Add 10 ml, 10× concentrated binding buffer.
- Adjust the pH to 7.4 using 1 M HCl.
- Add distilled water and makeup to 100 ml.

NB: Paraformaldehyde solution must be freshly prepared on the day of use.

4.1.5 Method for Annexin V Apoptosis Assay

There are two main methods for monitoring cell death using Annexin V.

Use of Flow Cytometry to Measure Phosphatidylserine.

Use of CSLM to Visualize Cell Surface Expression of Phosphatidylserine.

Here is a brief discussion about the above procedures:

4.1.6 Quantification of Phosphatidylserine Expression Using Flow Cytometry

- For positive control, take **1×10^6 cells ml^{-1}** and make a cell suspension in an appropriate medium.
- For positive control, add 5 μM **ionomycin** and incubate at 37 °C for 10 min.
- Of note, ionomycin is a calcium ionophore that enhanced PS expression on the cell surface by increasing the intracellular calcium concentration.
- For the experimental sample also, take **1×10^6 cells ml^{-1}** and make a cell suspension in an appropriate medium.
- Incubate the cells at 37 °C and induce apoptosis by adding an apoptosis-inducing experimental agent (e.g., 200 ng ml^{-1} antibody to Fas).
- At the predetermined time point, take 50 μl **(5×10^4) cells** and add with 450 μl binding buffer.
- Add 250 ng·ml^{-1} (each) green fluorescent Annexin V and propidium iodide.
- Incubate the mixture in the dark for 5 to 15 min at 20 to 25 °C (ambient temperature) or on ice.
- The incubation of either ice or ambient temperature depends upon the research question.

NB: Two-color flow cytometry is needed. The binding of green fluorescent Annexin V at the cell surface and propidium iodide uptake can be measured without the necessity of including a washing step.

- If washing steps are required, the wash buffer should contain (1 to 3) mM $CaCl_2$. The presence of Ca^{2+} ensures that the cell-bound Annexin V remains intact during the washing procedure. The final washing step should also include 250 ng·ml^{-1} propidium iodide.
- Analyze the flow cytometry data "offline." Set the quadrants in the green fluorescent Annexin V-versus-propidium iodide dot plot of the untreated control cells.

The respective locations for living cells, Annexin V binding, and propidium iodide positive cells are the bottom left quadrant, bottom right, and top right quadrants, respectively (Vermes et al. 1995; Poot and Pierce 1999).

4.1.7 Visualizing Phosphatidylserine Cell Surface Expression Using CSLM

- Make a cell suspension of $\mathbf{1 \times 10^6\ cells \cdot ml^{-1}}$ in an appropriate medium.
- Incubate the cells at 37 °C and induce cell death by adding a 200 $ng \cdot ml^{-1}$ antibody to Fas or other apoptosis-inducing agents.
- Following incubation, take a microfuge and add 5×10^5 $cells \cdot ml^{-1.}$
- Spindown the cells at 200 x *g* for 2 min.
- Remove the supernatant.
- Resuspend the cell pellet in a 500 μl binding buffer.
- Now, add 250 $ng \cdot ml^{-1}$ propidium iodide, and 250 $ng \cdot ml^{-1}$ fluorescence labeled Annexin V and incubate for 5 to 15 min at ambient temperature or on ice, depending on the research question.
- Again spin the cells at 200 x *g* for 2 min.
- Remove the supernatant, resuspend cells in 500 μl, 4% paraformaldehyde in binding buffer (the fixation buffer should be freshly prepared), and incubate for 15 min at an ambient temperature.

NB: Cell samples should not be fixed with paraformaldehyde before incubation with fluorescence-labeled Annexin V because it is likely to generate false-positive results.

- The paraformaldehyde buffer solution should contain $CaCl_2$ in the 1 to 3 mM concentration range.
- Place the 15 μl cell suspension on a glass slide before putting a glass coverslip over the cell suspension. Seal the glass coverslip over the glass slide using nail polish and analyze the cells by CSLM using wavelength excitation at 488 nm (for green fluorescent Annexin V) and 568 nm (for propidium iodide).

4.2 DNA Condensation and Fragmentation Assays

During the process of apoptosis, specific proteolytic enzymes, namely, caspases, are activated which again activate DNase. This leads to an oligonucleotide-sized fragment which can be detected via DNA electrophoresis. The ruined DNA exhibits a characteristic pattern of "**ladder**" formation. The major drawback of this technique is that it is not possible to quantify the number of cells that perished. This is a time-consuming technique.

On the other hand, the **TUNEL** (Terminal dUTP nick-end labeling) assay is a fast and sensitive technique. It allows the researchers to identify the fragmented DNA at the single cell stage. The first step in this method is to prepare the labeled dUTP at the 3′-end of DNA fragments using terminal deoxynucleotidyl transferase. In the next step, the fluorescent-labeled DNA can be analyzed either using flow cytometry

or fluorescent microscopy with Ex/Em 488/520 nm. The TUNEL assay is both fast as well as sensitive (Collins et al. 1997; Crowley et al. 2016).

4.2.1 Equipment and Materials for TUNNEL Assay

Flow cyto meter.
Phosphate-buffered saline (PBS), pH 7.4.
1% Formaldehyde in PBS, pH 7.4.
70% ethanol.
TdT (Boehringer Mannheim, Indianapolis, IND).

4.2.2 TDT Reaction Buffer (5X)

1.25 $mg \cdot ml^{-1}$ BSA.
1 M sodium or potassium cacodylate.
125 mM HCl, pH 6.6.

4.2.3 BR-dUTP Stock Solution (50 Ml)

Make 50 mM Tris-HCl, pH 7.5.
Add 2 mM Br-dUTP.
Add 10 mM $CoCl_2$.

4.2.4 Buffer for Rinsing

Take phosphate buffer saline (PBS).
Add 0.1% Triton X-100.
Add 5 $mg \cdot ml^{-1}$ BSA .
Mildly heat to dissolve.

4.2.5 Alexa Fluor 488 or FITC-Conjugated Anti-BR-DU Monoclonal Antibody

Take 100 µl PBS.
Dissolve 0.3 µg fluorochrome-conjugated anti Br-dU Ab.
Add 0.3% Triton X-100.
Add 1% (w/v) BSA.
Dissolve.

4.2.6 Preparation of Propidium Iodide Staining Buffer

In PBS, add the following:
5 $\mu g \cdot ml^{-1}$ PI,
100 $\mu g \cdot ml^{-1}$ RNase A (DNase-free).

4.2.7 Other Materials (To Be Used in Conjunction with LSC)

Microscope slides, Coplin jars, Glycerol, and Parafilm "M" or $\sim (2 \times 3)$ cm nylon foil strips.

4.2.8 Method for TUNNEL Assay

- Make a cell suspension by adding **1 to 2 × 10^6 cells** in 0.5 ml cold PBS.
- Take a 5 ml polypropylene tube.
- In this tube, add 4.5 ml ice-cold, 1% formaldehyde in PBS, and 0.5 ml of the above cell suspension.
- Mix by tapping.
- Keep the tube on ice for 15 min.
- Centrifuge at 300 x *g* for 5 min.
- Add 5 ml PBS to the pellet and resuspend it.
- Again centrifuge at 300 x *g* for 5 min.
- Again resuspend the cell pellet in 0.5 ml PBS.
- In a tube, add 4.5 ml of ice-cold 70% ethanol and 0.5 ml of the above cell suspension.
- Mix them.
- If necessary at this stage, the above sample can be stored for several weeks at −20 °C.
- To remove the ethanol, centrifuge at 200 x *g* for 3 min.
- Resuspend the cells in 5 ml PBS and centrifuge again at 300 x *g* for 5 min.
- Again resuspend the pellet in a 50 µl solution containing the following:
  10 µl TdT 5X reaction buffer.
  2.0 µl Br-dUTP stock solution.
  0.5 µl (12.5 units) TdT.
  5 µl $CoCl_2$ solution.
  33.5 µl distilled H_2O.
- In this solution, incubate the cells at 37 °C for 40 min.
- To rinse the cells, add 1.5 ml rinsing buffer, and centrifuge at 300 x *g* for 5 min.
- Add 100 µl FITC- (or Alexa Fluor 488)-conjugated anti-Br-dU-mAb solution and resuspend the cell pellet.
- Incubate at room temperature (RT) for 1 h.
- Add 1 ml PI staining solution.
- Incubate for 30 min at room temperature, or 20 min at 37 °C, in the dark.
- Analyze the cells using flow cytometry.

NB: In flow cytometry, three different fluorescent lights are used. They are red, green and blue. The red fluorescence of PI is measured at > 600 nm. The green fluorescence of FITC-(or Alexa Fluor 488)-anti-Br-dU Ab can be measured at 530 ± 20 nm. The blue fluorescent light can be measured at a 488 nm laser line or BG12 excitation filter.

4.3 Caspase Activation and Detection Assays

- Caspases are aspartate-directed cysteine proteases, present in the cytoplasm that cleave a diverse group of intracellular substrates and contribute to manifold apoptotic manifestations.

- These proteases are synthesized as inactive precursors and are activated as a consequence of signaling induced via a wide range of physiological and pathological stimuli.
- Caspases such as caspase 8 are activated either by extrinsic apoptotic signaling molecules such as TNFα or intrinsic apoptotic signaling molecules such as cytochrome-C, released from mitochondria which activate caspase 9.
- Extrinsic or intrinsic signaling cascades finally activate caspase 3, the executioner of apoptosis.
- In fact, in response to an apoptotic signal, cleavage of inactive caspase-3 occurs mainly at the Asp175 residue following which activation happens (Gurtu et al. 1997; Zhang et al. 2013).

Caspase activation can be detected by the following procedures:

Catalytic Activity Measurement
Immunoblotting for Cleavage of the Substrates
Use of Conformation-Sensitive Antibodies for Immunolabeling
Flow Cytometry or Ligand Blotting After Affinity Labeling

4.3.1 Equipment and Materials for Detecting Active Caspase 3 by Immunocytochemistry

Glass coverslips (22 mm × 22 mm) and slides.

4.3.2 4% Formaldehyde

Every time, make a fresh stock by diluting 37% formaldehyde to 4% in PBS.

4.3.3 Blocking Buffer

Take PBS and add the following:

2% goat serum.
0.3% Triton X-100.
Filter sterilize.

4.3.4 Primary Antibodies

Anti-active caspase-3.

4.3.5 Secondary Antibody

Fluorochrome-conjugated.
For fluorescence, vectashield mounting medium with or without **DAPI (**4′,6′-diamidino-2-phenylindole hydrochlorides) should be used.

4.3.6 Detection of Caspase 3 Protein Using Immunocytochemistry

- Sterile glass cover slips are used for plating the cells.
- To make the coverslips sterile, dip them in ethanol and then pass them through the flame.

- These sterile cover slips should be put into the 6-well plates, **1×10^5 cells/well** should be seeded per well and grown overnight.
- The next day, remove the medium and wash the cells with warmed (37 °C) PBS.
- To fix the cells, add 1 to 2 ml, 4% formaldehyde per well, and incubate for 20 min at room temperature.
- Wash the cells thrice with PBS, taking 5 min for each wash.
- To block the cells incubate them with blocking buffer for 5 to 10 min at room temperature.
- Now the cells are ready to be treated with primary antibodies. The antibody should be diluted in 100 to 200 μl blocking buffer, or as recommended by the manufacturer.
- ***NB: Two primary antibodies could be added at the same time, but they should have distinct origins (*e.g.*, one rabbit and another mouse)***.
- For treatment with the diluted antibody, use a different 6-well dish. Cut 3 cm diameter circle filter paper. Soak them in PBS and put them into each well of the 6-well plate. This will prevent the drying of the cells.
- Now put the coverslip on top of the filter paper.
- In the next step, carefully add diluted antibody to the entire coverslip surface and incubate at room temperature for 1 to 2 h.
- Following antibody addition, incubation is done to wash each well with PBS, three times, for 05 min each.
- Now add fluorochrome-conjugated secondary antibody to the blocking buffer and incubate for 30 to 45 min at room temperature in the dark.
- In the case of dual staining, the fluorophores used should have different emissions spectra for each specific antibody (e.g., FITC at 525 nM and phycoerythrin at 578 nM).
- Wash each well thrice with 1× PBS for 5 min at room temperature.
- Take the help of a forceps, and tilt the cover slip to drain off the excess PBS from the coverslips.
- For mounting, add a drop of Vectashield.
- Use Kim-wipe to remove excess Vectashield and now seal with nail polish.
- A similar protocol could be used for tissue sections (**Immunohistochemistry**).
- The slide can be used immediately or if necessary, can be stored in a − 20 °C freezer.

NB: Keep the slides in the dark, at all instants after adding the secondary antibody.

4.3.7 Colorimetric Assay for Caspase 3 Activity Determination

Equipment and Materials for Caspase 3 Activity Determination Using Colorimetric Assay

Instruments
Microtiter plate reader/spectrophotometer/or fluorometer.

Lysis Buffer
1% NP40, 20 mM HEPES (pH ~ 7.5), 4 mM EDTA. Just before use, add the following protease inhibitors: aprotinin (10 g·ml^{-1}), leupeptin (10 g·ml^{-1}), pepstatin (10 g·ml^{-1}), and phenyl methyl sulfonyl fluoride (PMSF) (1 mM).

Reaction Buffer
This buffer has the following composition:

5 mM DTT (Dithiothreitol).
0.5 mM EDTA,
20% v/v glycerol, and
100 mM HEPES, pH 7.5.

Caspase 3 Substrate
Some molecules that are used as caspase 3 substrates are as follows:

- Ac-DEVD-p-nitroanilide (**Ac-DEVD-pNA**).
- 7-amino-4-methylcoumarine (**AMC**).
- 7-amino-4-trifluoromethylcoumarin (**AFC**).

4.3.8 Method of Caspase 3 Activity Determination by Colorimetric Assay

In general, to measure the chromogenic group release from the synthetic substrate, the colorimetric assay is used. The best example may be the use of pNA (ADEVD-pNA) by activated caspases. The absorbance is recorded within (405 to 410) nM.

However, one of the major drawbacks of the ADEVA-based substrates is that these substrates are cleaved by most of the caspases, although caspase 3 is the most efficient in this context.

Several other DNA-based substrates are available for several caspases.

The activity of the caspase 3 can be determined by the following steps:

- Take **1×10^6 cells**.
- Wash with cold PBS.
- Resuspend the cells in 50x buffer.
- Vortex for a few seconds.
- Incubate on ice for 30 min.
- Centrifuge the cell lysate at 12,000 × *g* for 10 min at 4 °C.
- Take a fresh tube(s) and collect the supernatant.
- Keep on ice.
- Measure the protein concentration for each sample.
- Now take a 96-well plate and add 20 to 50 µl cell lysate, and caspase substrate (100 M final concentration to attain a final reaction volume of 200 µl.
- Incubate the reaction mixture at 37 °C for 1 to 2 h.
- Finally, monitor the enzyme-catalyzed pNA release at 405 nM using a microtiter plate reader.

4.3.9 Detection of Caspase 3 Activation by Immunoblotting

Equipment and Materials for Caspase 3 Determination

Instruments
Gel electrophoresis apparatus and reagents, Western blotting apparatus and reagents (TBST/PBST), Nitrocellulose or PVDF membrane, Milk (nonfat dry milk)/BSA as blocking agent.

Cell Lysis Buffer
This buffer has the following composition:

150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% NP-40, the protease inhibitor cocktail (1 mM PMSF and 1 $g \cdot ml^{-1}$ leupeptin) and 20 mM HEPES, pH 7.5.

Protein Measurement
BCA reagent and Standard BSA.

Primary Antibody
Active caspase-3 and PARP-1 antibodies.

Secondary Antibody
In general, antimouse or antirabbit antibodies are preferred.

Chemiluminescent Reagents
Lumiglo (KPL Inc., MD, USA) or ECL (Amersham).
Disuccinimidyl suberate (DSS), 2 mM final concentration.

Conjugation Buffer
20 mM sodium phosphate, pH 7.5 containing 0.15 M NaCl, 20 mM HEPES, pH 7.0, and 100 mM carbonate/bicarbonate, pH 9.0.

Quenching Buffer
1 M Tris-HCl, pH 7.5.

4.3.10 The Immunoblot Technique to Detect Caspase 3

As discussed previously, all the caspases are initially synthesized as inactive procaspases. During apoptotic activation, individual caspases are cleaved to generate the active caspases.

- Caspase 3 which is recognized as the final executioner of apoptosis, is synthesized as a 32 kDa inactive protein.
- During activation, the inactive procaspase 3 is converted to active caspase 3 (p17 and p12).
- PARP-1 is recognized as one of the important caspase 3 cellular substrates.

- In the apoptotic cells, caspase 3 cleaves the PARP1 (110 kDa) into two fragments, the most prominent of which is a 86 kDa protein.

The immunoblotting technique can be performed in the following steps:

- Following culture and treatment, harvest the cells and centrifuge at 500 × *g* for 5 min.
- *NB: For the immunoblot experiment, 1 × 10^6 cells may be used.*
- For washing the cells, decant the supernatant, resuspend the cell pellet in cold PBS and gently spin it down.
- The washing may be repeated 2 to 3 times.
- Remove all the PBS.
- For lysis, add the cold lysis buffer, and incubate on ice for 30 min with intermittent vortexing for a few seconds each time.
- Centrifuge the lysed cell material 15,000 × *g*, 15 min at 4 °C.
- The collected supernatant is the total cell lysate and the pellet is the cell debris.
- Discard the cell debris.
- Estimate the protein concentration of the supernatant. Several techniques of the protein concentration are there such as Lowry, BCA, Bradford, etc. However, Bradford is the widely used technique that measures protein by taking the absorbance at 595 nm. In this case, standard BSA with known concentration is generally used to calculate the concentration of the unknown protein sample.
- Prepare an (8–12)% gradient SDS-PAGE gel.
- Load 50 to 100 μg protein. In a separate lane, load standard protein with known molecular weight and run the gel at constant volt (to decrease the heat generation).
- Transfer the protein in PVDF/nitrocellulose membrane, either by semidry, dry, or wet technique.
- Block the membrane with either 5% milk or 5% BSA for 1 h at room temperature or overnight at 4 °C.
- Follow the manufacturer's recommendation to incubate the membrane with a primary antibody against **PARP-1** and **active caspase 3**. Generally, for room temperature, the incubation time is 2 to 3 h and for 4 °C the incubation time is overnight.
- Three washes with **PBST** (phosphate buffer saline and tween 20) or **TBST** (Tris buffer saline and tween 20) for 10 min, should be given, each at room temperature.
- Add appropriate secondary antibody with appropriate dilution (according to manufacturer's protocol).
- Of note, based on the primary antibody species, the secondary antibody should also be raised in the same species. For example, if the primary antibody is raised in the mouse, the secondary antibody must be raised in the mouse only.
- Following secondary antibody treatment wash the membrane thrice with PBST or TBST.
- Wash the blot with double distilled water to get rid of unbound tween 20.

- Finally, develop it using chemiluminescent reagents such as lumiglo or ECL, following the manufacturer's suggested protocol.

4.4 Cytochrome C Release Assay

- **Cytochrome C** is a heme protein localized in the compartment between the inner and outer mitochondrial membranes where it transfers electrons between complex III and complex IV of the respiratory chain.
- Cytochrome C binds to **cardiolipin** in the inner **mitochondrial membrane**, thus anchoring its presence and keeping it from getting released out of the mitochondria and initiating apoptosis.
- Cytochrome C has a primary structure consisting of nearly 100 amino acids chain. Many higher-order organisms possess a chain of 104 amino acids.
- As and when mitochondria receive apoptotic stimuli, the cytochrome C would be released into the cytoplasm.
- In presence of dATP, it causes allosteric activation of the adaptor molecule namely apoptosis protease activating factor 1 (**Apaf-1**). This leads to the generation of **apoptosome complexes**.
- **The function of the apoptosome is to recruit and activate caspase 9**. **Each apoptosome can activate and recruit up to seven caspases 9 dimers**.
- Finally, this activation leads to proteolytic self-processing (Waterhouse and Trapani 2003).

4.4.1 Equipment and Materials for Cytochrome C Release Assay

- V-bottomed 96-well plate.
- Mammalian cells.
- Apoptotic stimulus (e.g., cytotoxic drugs).
- Electrophoresis apparatus for SDS-PAGE.
- PVDF/Nitrocellulose membrane transfer apparatus.
- All chemicals for Immunoblot/Western blot.

4.4.2 Plasma Membrane Permeabilization Buffer

Take PBS buffer.

Add 200 $\mu g \cdot ml^{-1}$ digitonin.

Add 80 mM KCl.

Mix both to obtain a homogeneous texture.

4.4.3 Buffer for Total Cell Lysis

50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.2% Triton X-100, 0.3% NP-40, 1X Complete Protease Inhibitor Cocktail (PMSF, Aprotinin, leupeptin).

4.4.4 Phosphate-Buffered Saline

8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4 \cdot 7H_2O$, 0.2 g KH_2PO_4/L, pH 7.3.

Paraformaldehyde (4% in PBS).

4.4.5 Blocking Buffer

3% BSA, 0.05% saponin in PBS (make fresh).

4.4.6 Antibody

Anticytochrome C antibody (clone 7H8.2C12, BD Pharmingen, San Diego, CA), anticytochrome C (clone 6H2.B4, BD Pharmingen, San Diego, CA).

4.4.7 Method of Cytochrome C Release Detection

The method can be divided into two major steps:

1. Isolation of Cytosolic and Mitochondrial Fractions.
2. Western Blot or Fluorescence-Activated Cell Sorting for the Detection of Cytochrome C.

Briefly, the steps are as follows:

4.4.8 Isolation of Cytosolic and Mitochondrial Fractions

The major steps involve the disruption of the plasma membrane (may be via homogenization), followed by differential centrifugation to fractionalize cytosol and mitochondria.

However, homogenization may disrupt a large number of mitochondria, and therefore, other milder techniques may be utilized.

In this case, **digitonin** or **streptolysin O** may be the preferred choice, since the concentration of these molecules to disrupt the plasma membrane does not affect the mitochondrial membrane.

Following the use of permeabilization buffer containing digitonin (composition discussed in the above paragraphs), separation of the cytosol from the mitochondria can be accomplished using a single centrifugation step.

So, briefly, the basic steps may be as follows:

- Resuspend **1 x 10^6 cells** in 100 μl ice-cold plasma-membrane-permeabilization buffer and incubate on ice for 5 min.
- Centrifuge lysates (800 g for 5 min at 4 °C).
- Store the supernatant (cytosolic fraction) at 70 °C (for Western blot, do not discard the pellet).

4.4.9 Detection of Cytochrome C by Western Blot

- Resuspend the above pellet in 100 μl ice-cold total cell lysis buffer and rock gently at 4 °C for 10 min.
- Centrifuge the lysate (**10,000 x g) for 10 min at 4 °C**.
- -80 °C ultra-deep freezer can be used to store the supernatant (mitochondrial or nuclear membrane fraction).
- Measure the protein concentration of the cytosolic extract and mitochondria.
- Run on a **12 or 15% SDS-PAGE** gel before transferring the proteins to the nitrocellulose membrane.

- Detect cytochrome C (**12.3 kDa**) with an anticytochrome C clone antibody using the standard immunoblotting protocol.

4.4.10 Detection of Cytochrome C Using Fluorescence-Activated Cell Sorting

- Resuspend the above pellet in 100 μl paraformaldehyde and incubate for 20 min at RT.
- Take a 96-well plate. Place the cell suspension. Wash the cell pellet in PBS three times.
- Incubate the pellet in a blocking buffer for 1 h at RT.
- Resuspend the pellet in anticytochrome C antibody diluted at 1:200 in the blocking buffer and incubate overnight at 4 °C.
- To remove nonbound/loosely bound and excess antibodies, wash the pellet in the blocking buffer.
- Repeat the washing thrice.
- Resuspend the pellet in phycoerythrin (**PE**)-labeled secondary antibody diluted at 1:200 in a blocking buffer and incubate for 1 h at RT.
- Wash the pellet and analyze PE fluorescence using flow cytometry in the FL-2 channel of a flow cytometer.
- Cells having undergone cytochrome C release are likely to generate low PE fluorescence.

NB: *Make a 20 mg·ml^{-1} digitonin stock by adding PBS just before it is needed and heat the mixture at 95 °C until thorough dissolution. The KCl concentration in the cytoplasm is approximately 137 mM.*

- To facilitate the cytochrome C release from the cytoplasm, at least 80 mM KCl is required.
- For each cell line, the amount of permeabilization buffer should be titrated separately. This would ensure that at least 95% of the cells are permeabilized.
- This can be monitored via trypan blue staining of the permeabilized cells' small aliquots.
- Ensure that the chosen digitonin concentration does not lyse the mitochondria.
- For the background cytochrome C release, the same number of untreated and intact cells could be used.
- The number of cells assayed can be varied as long as the digitonin concentration is titrated.
- In permeabilized cells, Ca^{2+} will access mitochondria, inducing permeability transition and swelling.
- The use of metal/Ca^{2+} chelating agent EDTA is recommended if the cell medium contains a high amount of Ca^{2+}.
- Many of the cells express a high amount of cytochrome C, which could be easily detected by Western blotting. It indicates that Western blot may not be useful for those cells which express a low concentration of cytochrome C (such as primary T cells).

- In the case of low cytochrome C in the cytosolic fraction, one can precipitate the cytochrome c using acetone. However, a high level of digitonin may interfere with the precipitation.
- To effectively determine the exact amount of cytochrome C, it is better to detect both the cytosolic and mitochondrial cytochrome C under all conditions.
- While Western blot provides the average results of the cytochrome C amount in the cells, the immunocytochemistry and FACS-based analysis provide the data of the individual cells undergoing cytochrome C release.
- The fluorescence of stained and unstained cells is easily distinguishable to usefully screen the cells for cytochrome C release, by the FACS analysis.

4.4.11 Comparison of Various Assays

- Analysis of the sequence of the apoptotic events revealed that activation of inactive caspases to the active regime is one of the most important early apoptotic events. One of the intermediate events in apoptosis is the flipping of the phosphatidyl serine from the inner layer to the outer layer of the plasma membrane. Another important hallmark of apoptosis is DNA fragmentation.
- Thus, apoptotic assays are presently used either via screening early events (the caspase activation), mid events (phosphatidyl serine level in the outer layer of the plasma membrane and late events (DNA fragmentation by TUNEL assay).
- The activated caspases can be detected either by Western blotting or flow cytometry.
- The TUNEL assay which detects the fragmented DNA in the nucleus is a time-consuming technique. Most importantly, some apoptotic cells do not display DNA fragmentation.
- It is now widely recognized that the use of Annexin V is one of the most versatile and effective techniques to determine various aspects and forms of apoptosis. It is further recognized that Annexin V conjugates with various fluorochromes that include fluorescein isothiocyanate, FP488, Alexa Fluor dyes, phycoerythrin, and carbocyanine. It is generally recommended to use Annexin V along with other techniques such as caspase assay or TUNEL assay to gather thorough knowledge about apoptosis.

5 Cell Attachment/Adhesion Assay

- Cell adhesion or binding of a cell to the extracellular matrix (**ECM**), other cells, or a specific surface (e.g., cell culture containers), is essential for growth and survival and also for its growth-sustaining communication with other cells.

Briefly, the process of cell adhesion involves the following three processes:

- Reorganization of the cytoskeleton.
- Altered biochemical reactions in the cells.

- Changes in the attachment molecules on the cell surface followed by interaction of the cell adhesion molecules with the counter receptors/ligands.
- By interacting with the various ECM proteins as well as receptor molecules on the surface of other cells, the adhesion molecules maintain cell-to-cell communication and cross-talk. ***This type of cross-talk is highly essential for the development and maintenance of the tissues in a complex mammalian body comprising of a multiorgan system***.
- Several lines of evidence indicate that for the in vitro cell culture of most mammalian cells (except some blood cells, e.g., **B/T lymphocytes**), adhesion/attachment with the cell culture vessel is essential. Thus, most mammalian cells are considered anchorage-dependent.
- However, some of the cells also exhibit a loose anchorage dependence. The more the number of chemical bonds on the cell's surface, the higher the chances of cell adhesion.
- The adherent cells cannot survive **in absence of cell adhesion or experimentally knocking out the complete expression of the adhesion molecules**.
- **However, adherent cells can slowly adapt to nonadherent or suspension cultures**.
- Experimental results have demonstrated that as cell adhesion gets stimulated it regulates the **cell cycle, cell differentiation, and migration**.
- **Under diseased conditions such as cancers and atherosclerosis, the expression of cell adhesion molecules acquires a very important role**.

The usefulness of the cell adhesion assay is as follows:

- To Test the Ability of a Specific Cell or Cell Line for Adherence to a Characteristic Adhesive Substrate.
- To Test the Sensitivity of Specific Cell–Substrate–Inhibitor Interactions or the Ability of Certain Experimental Chemicals/Drugs to Affect the Cell Adhesion.
- The Assays Can Be Used to Probe the Contribution of Other Cellular Processes Responsible for Sustaining Metastatic Ability of Cancer Cells (Humphries 1998; Mobley and Shimizu 2001; Liang et al. 2007; Chen et al. 2009; Justus et al. 2014; Khalili and Ahmad 2015).

5.1 Equipment and Materials for Cell Adhesion Assay

- HeLa cells.
- Corning 96-well polystyrene plate.
- A cell culture incubator, set at 37 °C and 5% CO_2.
- A spectrophotometer (ability to reveal absorbance as a 96-well plate).
- MTT cell proliferation assay kit.
- Dulbecco's Modified Eagle Medium (DMEM).
- 0.1% BSA solution in DMEM.
- Fetal Bovine Serum (FBS).
- EDTA solution (0.5 M, pH 8.0).

- Bovine Serum Albumin (BSA).
- Phosphate-Buffered Saline (PBS).
- Collagen 1 solution in PBS (40 $\mu g \cdot ml^{-1}$), store at 4 °C.

5.2 Detection Methods of Cell Adhesion

- "Grow the HeLa cells in DMEM supplemented with 10% FBS.
- Coat the 96-well plate (30 µl/well) with the Collagen I solution at 4 °C.
- After a 12-h coating, remove the Collagen I solution and air-dry the plate at RT in the tissue-culture hood.
- Deprive cells of serum for 8 h before the adhesion assay.
- To do so, wash the cells thrice with serum-free DMEM and grow them in DMEM.
- Use 10 mM EDTA in DMEM to detach the cells before observing them under a microscope to confirm complete dissociation, which would take ~10 min.
- Wash the cells twice with DMEM to remove EDTA, and resuspend the cells at 2 x 10^5 cells per ml in DMEM supplemented with 0.1% BSA.
- For cell–substratum adhesion assay, add 100 µl cell suspension to each of the Collagen I-coated wells. Incubate the plate at 37 °C for 20 min to allow the surface adherence of the cells.
- Add 100 µl DMEM to each well to get rid of any nonadherent cells, wash four times."

NB: To achieve consistency, always add/remove DMEM gently with a multichannel pipette for multiple wells.

- After washing, add DMEM with 10% FBS and incubate the cells at 37 °C for 4 h to achieve recovery.
- Add 10 µl MTT substrate to each well and continue incubation for an additional 2 h at 30 °C.
- Next, lyse the MTT-treated cells in 100 µl DMSO (or another lysis buffer of choice) and measure absorbance at 570 nm on a spectrophotometer.

6 Cell Migration Assay

Cell migration could be ascertained using several methods including scratch assays, cell-exclusion zone assays, microfluidic-based assays, and Boyden Chamber assays.

The two methods generally used for mammalian cell migration assay are as follows:

1. In Vitro Scratch Assay.
2. Boyden Chamber Assay.

The following paragraphs discuss these two assays:

6.1 In Vitro Scratch Assay

- The in vitro scratch assay is a straightforward and economical method to study cell migration in vitro.
- Since the scratch or wound created on the cell culture is gradually filled by the growing migrating cells, this technique is also called **Wound Healing Assay or Scratch Wound Healing Assay**.

The scratch wound healing assay involves the following three steps:

Make a Confluent Monolayer of Cells.
Make an Artificial Gap/Scratch at the Center of the Culture Vessel, Take Images and Then Incubate.
Closely Watch from Time to Time While the Scratch Is Filled with the Migrating Cells. Compute the Migrating Rate (Liang et al. 2007; Jonkman et al. 2014).

Here are some advantages and disadvantages of in vitro *scratch assay:*

6.1.1 Advantages of In Vitro Scratch Assay

- In vitro scratch assay is often the method of choice to analyze cell migration in a laboratory because of its simplified setup, no requirement of specialized equipment, and easier availability of all materials required for the cell culture assay. Thus, **it is the simplest and least expensive technique**.
- To some extent, it mimics in vivo cell migration. For example, the removal of the endothelium in the blood vessels induces the migration of endothelial cells (**ECs**) into the denuded area to enclose the wound.
- Not only the strictly adherent endothelial cells, but the loosely adherent cells such as fibroblasts or cells that behave like a sheet such as epithelial cells also mimic a similar kind of behavior amidst cell migration in the in vivo conditions.
- This assay is particularly suitable to study the cell migration regulation by cell-extracellular matrix (ECM) and cell–cell interactions.
- **Live cell imaging of the assay** would simplify the understanding of cell signaling events. Visualization of the **GFP** (Green Fluorescent Protein) tagged proteins for subcellular localization or **FRET** (Fluorescent Resonance Energy Transfer) for protein-protein interaction during cell migration, is possible.
- In vitro scratch assay can be combined with other techniques, such as **microinjection or gene transfection**, to assess the effects of exogenous gene expression on individual cell migration.
- At present, using **time-lapse microscopy and image analysis software**, one can track the migration pathway of the individual cell across the leading edge of the scratch.
- The role of the individual gene with their protein product in the cell migration can be determined using this assay.

6.1.2 Disadvantages of Scratch Assay

- The assay requires completion in Petri plates/cell culture dishes. So, it needs a large number of cells and culture a medium to fill the culture dish. Therefore, some of the primary cultured cells which are hard to get in large quantities or any expensive chemicals that are needed for cell culture are not suitable for this assay.
- Using the scratch assay, no chemical gradient can be established.
- It is a long-duration procedure since the completion of the whole procedure takes a couple of days. It takes at least one to 2 days to form the cell monolayer and then again one to 2 days, to fill the scratch (Chen 2005).

6.1.3 Equipment and Materials for Scratch Assay

- Cell culture incubator conditions: 37 °C and 5% CO_2.
- 24-well tissue culture plate.
- Any cancer cell line such as MCF-7 breast cancer cells.
- Phosphate-Buffered Saline (PBS).
- Dulbecco's Modified Eagle Medium (DMEM).
- Fetal Bovine Serum (FBS).
- Glutaraldehyde.
- Ethanol.
- Crystal violet.

6.1.4 Scratch Assay-Mediated Determination of Cell Migration

- Grow MCF-7 cells in 10% FBS supplemented DMEM.
- Seed the cells into a 24-well tissue culture plate at a density that could enable **70** to **80%** confluence (as a monolayer) after **24 h** of growth.
- Before the cell culture assay, no medium change is required.
- Take a new, sterile 1 ml pipette tip and make a scratch at the center of the well. The scratch should be in one direction only.
- While making the scratch, the long-axial of the tip should always be perpendicular to the bottom of the well. Therefore, the gap distance in the scratch should be equal to the outer diameter of the scratch. If necessary, the gap distance can be adjusted using a different size of the tip.
- Perpendicular to the first scratch, a second scratch can be created to finally be displayed as a **cross-like structure**.
- Remove the detached cells from the scratch by gently washing twice with the culture medium.
- Now add fresh medium to the cells and allow the growth for 24 to 48 h or as required by the experimental design.
- Wash with PBS and fix with 3.7% paraformaldehyde for 30 min.
- Stain the fixed cells with 1% crystal violet in 2% ethanol for 30 min.
- Following staining, take photographs on a microscope. The configuration of the microscope cannot be changed while taking different photographs.
- **"The gap distance can be quantitatively evaluated using software such as Photoshop or ImageJ (http://rsb.info.nih.gov/ij/download.html)."**

- **NB**: It is suggested that to reduce the variability of the results, multiple photographs from each well should be taken before analysis.

NB: *Medium may contain ingredients of interest that warrant testing,* e.g.*, chemicals that inhibit/promote cell motility and/or proliferation*.

- The rate of migration can be calculated based on the available software.
- The nonmigrating neighboring cells can be considered as controls.
- Sometimes, for the betterment of calculation, an imaginary line can be drawn in the middle of the scratch.

6.2 Boyden Chamber Assay

- **Boyden chamber assay, originally introduced by Boyden** is recognized as one of the most widely accepted cell migration assays. At present, this assay is nicknamed **transwell migration assay, filter membrane migration assay, or chemotaxis assay**.
- In the original assay, Boyden analyzed leukocyte migration (**chemotaxis**) with the help of a cell culture medium-filled hollow plastic chamber which is sealed at one end with a porous membrane. This chamber is suspended over a large well which may contain cell culture medium/chemoattractant.
- Commercially, several variations of the Boyden chamber are available. In general, a large cell culture medium-filled chamber is separated into two compartments using a microporous membrane.
- In this procedure, cells are seeded in the upper compartment and allowed to survive, grow, and finally migrate into the lower chamber that may have chemotactic agents (Fig. 5).
- After a designated incubation time, the membrane between the two compartments is fixed and stained to determine the number of cells that migrated to the lower side of the membrane.
- *NB: Selection of the pore diameter is very important for completing the migration assay. At present in the Boyden assay, 3 to 12 μm pore diameter is generally used. Most mammalian cells having a size of 30 to 50 μm, can migrate across the 3 to 12 μm pore diameter. However, lymphocytes having a size of 10 μm can migrate through pores as small as 0.3 μm*.

Here are some examples of cell-specific optimized pore sizes.

- 3 μm pore size is appropriate for the leukocyte or lymphocyte migration.
- 5 μm pore size is appropriate for a subset of mouse fibroblast cells (NIH-3 T3 cells), or triple-negative human breast cancer cells (MDA-MAB-231).
- This 5 μm pore size limit is also suitable for **monocytes** and **macrophages**.
- As mentioned earlier, the 8 μm pore size may be suitable for most cells, but not for leukocytes.

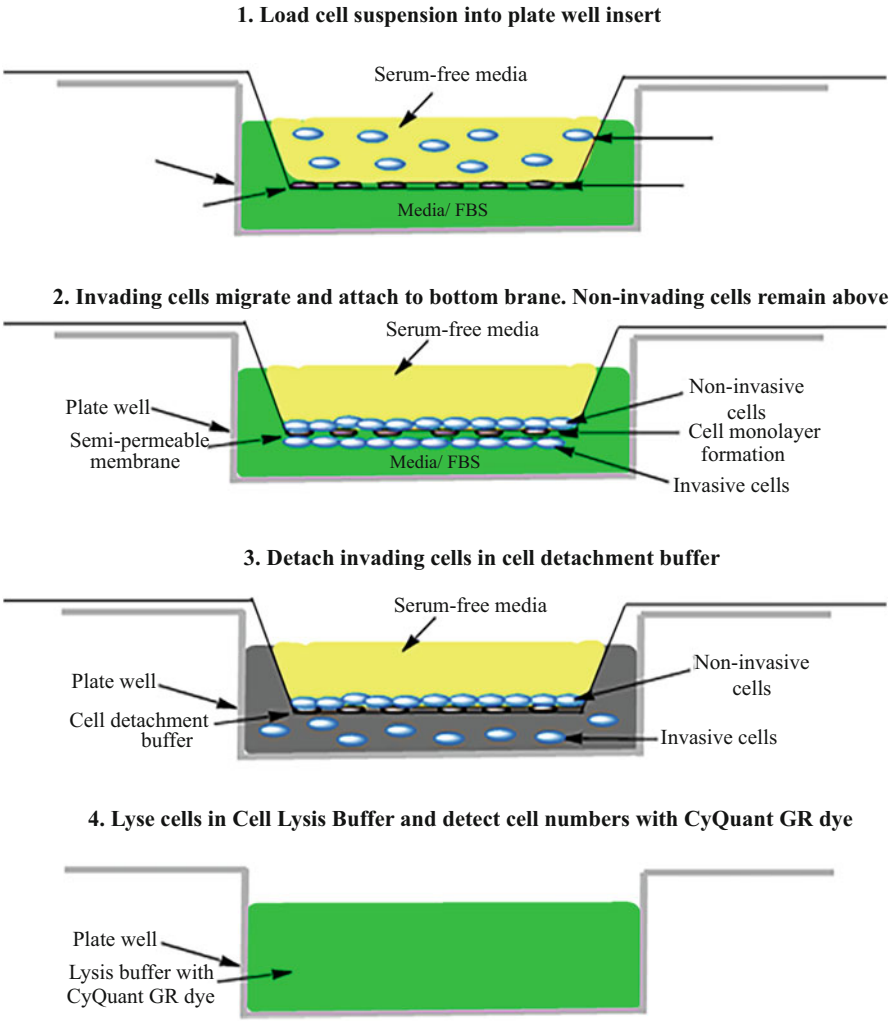


Fig. 5 Chronological steps of a Boyden Chamber Assay

6.2.1 Drawbacks of Boyden Chamber Assay

In the Boyden chamber assay, cells are prepared in suspension before performing the assay. This disrupts cell–cell and cell–ECM interactions.

7 Measurement of Reactive Oxygen Species

- Reactive oxygen species (**ROS**) is a collective term for different reactive molecules and free radicals ($\mathbf{10^9–10^{12}}$ **sec**) derived from molecular oxygen. Oxygen in its ground state has two unpaired electrons ($\mathbf{1s^2\ 2s^2\ 2p^4}$). So, it is easy for oxygen to accept electrons and form free radicals.

- *"The most common ROS include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), and singlet oxygen ($^1O_2$), all of which are more reactive than oxygen (O_2) itself."*
- Virtually, even in normal physiological conditions every mammalian cell constantly generates ROS at a low basal level. In a mitochondrial electron transport chain, the electron is carried by FAD and NAD from complex I to complex IV via complex II and complex III, finally causing oxidative phosphorylation. This ROS is produced by the leakage of an electron from the complex III of the mitochondrial electron transport chain, leading to a **single electron reduction**.
- However, defensive phagocytic cells such as macrophages produce a large amount of ROS to eliminate various pathogens. The reactive molecules are produced during the normal electron transport of mitochondrial aerobic respiration (leakage of an electron from the complex III of mitochondrial electron transport chain (**ETC**), **a single electron reduction,** and **ROS generation**) or by the normal activity of oxidoreductases (e.g., phagocytic NADPH oxidase (Phox)/nonphagocytic NADPH oxidase (Nox), xanthine oxidase, other oxidoreductases, and metal-catalyzed oxidations.
- The reduction of molecular oxygen (O_2) produces superoxide ($O_2^{\bullet-}$), the precursor of most other ROS,

$$O_2 + e^- \rightarrow {}^{\bullet}O^-$$

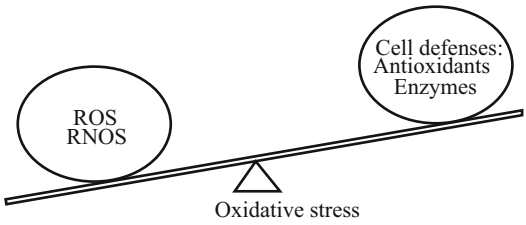
Dismutation of superoxide generates hydrogen peroxide (H_2O_2):

$$2\,H^+ + {}^{\bullet}O^-$$
$$2 + {}^{\bullet}O^-$$
$$2 \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide, in turn, may be partially or fully reduced to hydroxyl radical ($^{\bullet}OH$) and water, respectively, as depicted in the following reactions:

$$H_2O_2 + e^- \rightarrow HO^- + {}^{\bullet}OH$$
$$2H^+ + 2e^- + H_2O_2 \rightarrow 2H_2O$$

The low level of ROS generated by the mammalian cells in the normal physiological processes helps in cell survival, proliferation, and setting up of oxidative defense mechanisms responsible for killing the bacteria and other pathogens besides regulating the various cell signaling events and physiological homeostasis. ROS is labeled as a secondary cell signaling **messenger** (Fig. 6).



Major oxygen metabolites prodcued by one electron reduction

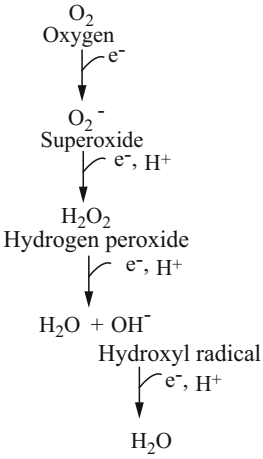


Fig. 6 Reduction of oxygen by four one-electron steps. The four one-electron reduction steps for O_2 consecutively generate superoxide, hydrogen peroxide, hydroxyl radical, and water

7.1 The High Level of Reactive Oxygen Species and Oxidative Stress

- Like the low level of ROS, a moderately increased ROS expression may not pose any risk for native physiology. This is because moderately high free radicals are scavenged by different antioxidant mechanisms in the mammalian cells such as glutathione (GSH, the most abundant reductive molecule in the human cell cytoplasm), thioredoxin, catalase, various superoxide dismutases (SODs), etc.
- The delicate balance between ROS generation and ROS scavenging is disturbed by the different stress factors like UV radiations, bacterial LPS, cytokines like TNFα, etc.
- The imbalance is generated due to a high level of ROS generation than what is compensated by the antioxidant molecules, leading to a pathophysiological condition, termed "**Oxidative Stress**."
- Oxidative stress is very important to mammalian pathophysiology because it complicates the pathology of almost every noncommunicable disease such as

cancers, cardiovascular diseases (**CVD**), diabetes mellitus, chronic obstructive pulmonary diseases (**COPD**), Alzheimer's disease, and several others.

- Therefore, detection of generated ROS is not only helpful toward understanding the ROS-dependent normal physiological cell signaling including activation of MAPKs and cell cycle leading to cell division but also in the oxidative stress-driven complicated mechanisms of diseased conditions such as CVD, COPD, and cancers. This also enhances the therapeutic implications of targeting ROS-generating molecules of the body.

7.2 ROS Detection Is Mainly Based on the Following Methods

Figure 7 outlines the various methods optimized for ROS detection, the brief characteristics of which are summarized ahead.

7.2.1 Spectroscopic Methods

The use of specific spin traps and probes forming paramagnetic adducts.

7.2.2 Fluorescent-Dependent Methods

Under the conditions of oxidative stress, the oxidation-sensitive probes exhibit an enhanced fluorescence.

7.2.3 Chemiluminescent Probes

In this case, ROS reacts with the probe leading to the generation of the photons, which are monitored.

7.2.4 Spectrophotometric Methods

Reactive species interact with antioxidant molecules and produce variations in absorbance.

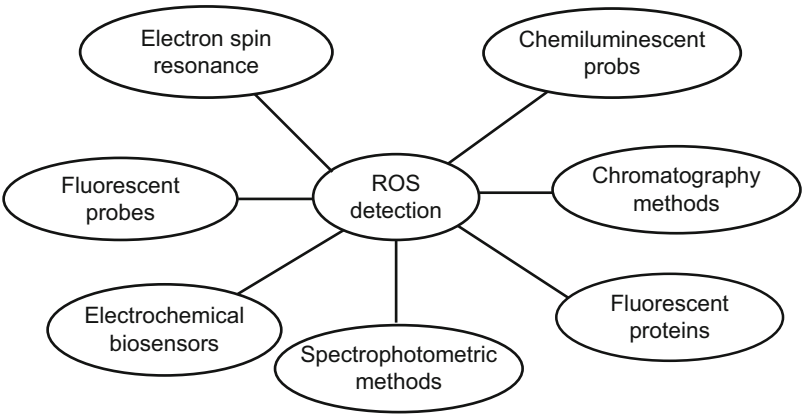


Fig. 7 Different methods of ROS quantification

7.2.5 Chromatographic Method

Separation and identification of reactive species and products are accomplished.

7.3 Electrochemical Sensors (Chip-Type Biosensors)

Upon generation of reactive oxygen species, there would be an alteration in oxidation /reduction balance, which would be monitored by electrochemical sensors.

Some of the ROS determination techniques are described as follows:

1. Determination of ROS Generation by 2′,7′-Dichlorofluorescein-Diacetate
2. Superoxide Measurement by Nitroblue Tetrazolium Method
3. ROS Measurement by Chemiluminescent Probes
4. ROS Measurement by Fluorescent Probes
5. Superoxide Detection by Cytochrome C Release
6. ROS Measurement by Immuno-Spin Trapping

Here is a brief discussion about them:

7.3.1 Determination of Reactive Oxygen Species by 2′,7′-Dichlorofluorescein-Diacetate

- The measurement of ROS is dependent on the analytical target(s) along with the ROS in question.
- ROS can be measured by screening the cultured cells. In the case of an animal model, ROS or oxidative stress products can be measured from blood plasma or urine.
- Several methods have been developed to measure various types of ROS.
- **While some methods directly measure ROS, other methods measure ROS-generated oxidative products**.
- However, since reactive oxygen products such as superoxide are stable for very less time, it is very challenging to **directly measure** these molecules.
- Thus, detection of ROS levels substantially relies on screening end products of their probable fates (**such as hydrogen peroxide or H_2O_2**), either by **chemiluminescence** or **fluorescence in** response to the reaction of specific compounds with ROS.
- At present, one of the most widely used probes is dichlorofluorescein-diacetate (**DCFH-DA**).
- Being able to enter into cells, the probe enters inside the cells and is henceforth, **hydrolyzed by intracellular esterases** to DCFH carboxylate anion which is retained inside the cell. Now oxidation of two electrons of DCFH results in the formation of a green fluorescent product, **dichlorofluorescein (DCF)**.
- Multiple fluorescence-based techniques such as **confocal microscopy** or **flow cytometry** as well as **fluorimeter** can be used to measure dichlorofluorescein.
- This is regularly used as a general technique to ascertain the titer of H_2O_2 and other oxidants. This technique can also be utilized to measure redox signaling

mediated cellular alterations in response to intra- or extracellular activation with the oxidative stimulus (Hafer et al. 2008; Dikalov and Harrison 2014).

Drawbacks of 2′,7′-Dichlorofluorescein-Based Reactive Oxygen Species Measurement

Though using H_2DCF is one of the most widely used methods for ROS detection, still several concerns have been raised in connection with this assay. Some drawbacks of using this method are as follows:

- ***"The intermediate radical, DCF•$^{-}$, formed from the one-electron DCFH oxidation rapidly reacts with O_2 (k = 108 M^{-1}•s^{-1}) to form superoxide ($O^{\cdot -2}$). This $O^{\cdot -2}$ dismutation yields additional H_2O_2, paving way for a redox-cycling mechanism leading to artifactual amplification of the fluorescence signal intensity."***
- In some cells, the hydrolyzed products may leak out of the cells. Under this condition, an alternative molecule such as chloromethyl derivative (**CM-H_2DCFDA**) is much better retention in live cells than H_2DCFDA.
- Besides H_2O_2, the dye can be oxidized by a host of ROS species such as **hydroxyl radicals, organic hydroperoxides, nitric oxide (NO),** and **peroxynitrite anions ($ONOO^{-}$)**.
- In the case of H_2DCF oxidation by H_2O_2, the reaction is significantly accelerated by the presence of peroxidases.
- It was also reported that H_2DCF oxidation depended on the concentration of glutathione (GSH), the most abundant antioxidant in mammalian cells.
- Thus, H_2DCF is perhaps, an indicator of the general oxidative stress extent.
- Besides live cells, even dying or dead cells also produce ROS but the probe is incapable of distinctive recognition of ROS generating sources from dead and live cells. Some reports claim that using PI could be substantial.
- Under proapoptotic conditions, mitochondria release a heme protein called **cytochrome C** which is capable of directly or indirectly oxidizing DCFH to DCH. Thus, ***"the increase in DCF fluorescence that occurs during apoptosis of cells loaded with DCFH-DA, is frequently associated with enhanced oxidant production."***
- Finally, it has been claimed that redox-active metals (e.g., Fe^{2+}) promote DCFH oxidation in the presence of oxygen or H_2O_2.

Equipment and Materials for ROS Determination Using 2′,7′-Dichlorofluorescein

- Cell incubator.
- Six-well plates/other sizes of cell culture containers.
- Centrifuge.
- FACS machine (Flow cytometer).
- 2 x 75 mm, 5 ml polystyrene round-bottomed test tube.
- Trypsin-EDTA (1x) (0.05%/0.02% in PBS).
- Dulbecco's Phosphate-Buffered Saline (DPBS) medium, without Ca^{+2} and Mg^{+2}.

- Hank's Balanced Salt Solution (HBSS) with Ca^{+2} and Mg^{+2} but without red phenol.
- CM-H_2DCF/CMH_2DCFDA.
- Propidium Iodide (PI) (1.0 mg·ml^{-1}).

NB: A probe fluorescence emission can be assessed using Flow cytometry, a standard fluorometer, or fluorescence microscopy using an appropriate filter.

- At present, the most commonly used technique to measure intracellular ROS is flow cytometry. The most significant aspect of this technique is that it allows the measurement of individual cells' fluorescence.
- In this technique, the cells are excited by the light source which emits light of varying wavelengths. In the next step, the emitted light is converted to electrical pulses by the optical detectors.
- The emitted light is scanned using the various detectors which fractionate it into constituent light waves of varying wavelengths. The terminology for the functioning is recognized as follows: **525 nm Band Pass Filter for FL-1 and 620 nm Band Pass Filter for FL-3**. The working of these filters is tuned by the placement of light filters in the passage of incident light. For example, a 525 nm band pass filter (FL-1) positioned in the light passage before the detector permits only green light incidence over the detector.
- Thereby, FL-1 functioning in the assay gathers green light for the ROS-driven dichlorodihydrofluorescein (DCF) oxidation. Usually, FL-1 infers a f = green channel on flow cytometers.
- Similarly, a 620 nm Band Pass Filter (FL-3) permits only "red" spectrum passage across the detector. The red fluorescence of emitted light is usually scanned in the FL-3 channel on the flow cytometers.

Method of ROS Determination Using 2′,7′-Dichlorofluorescein

- For screening, the ROS generation and its corresponding quantification, in general, primary cultured human umbilical vein endothelial cells (**HUVECs**), are used.
- These cells are treated with **10 ng·ml^{-1} TNFα** for **zero (0), 1 min, 30 min, 1 h, 3 h,** and **10 h**.
- One can also use other cell lines such as MCF-7 breast cancer cells and also other ROS-producing agents such as bacterial cell wall constituent lipopolysaccharide **(LPS)**.

Here is the protocol for ROS measurement:

- Culture **2.5 × 10^5 HUVECs** cells per well into 6-well gelatin-coated plates in a complete M199 medium and incubate in a CO_2 incubator overnight.
- The following day, as and when the cells reach around 80% confluency, the medium is changed to phenol red-free, growth factor, and sodium pyruvate free with a low serum content. Cells are treated with **10 ng·ml^{-1} TNFα** for various time points (zero, 1 min, 30 min, 1 h, 3 h, and 10 h.).
- For each time point (e.g., 1 min), all the 6 wells of a 6-well plate are used.

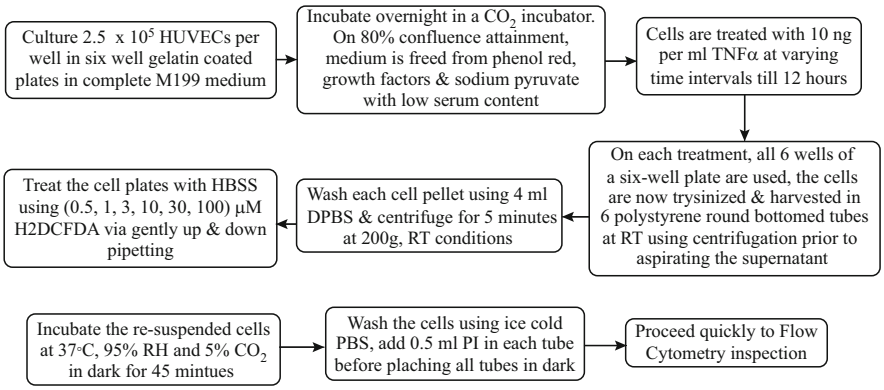


Fig. 8 Flow sequence of screening the generated ROS extent

- Following the treatment, the cells are trypsinized and harvested into 06 different 5 ml polystyrene round-bottomed tubes by centrifugation (5 min at 200 x *g*) at RT before aspirating the supernatant.
- Wash each cell pellet with 4 ml DPBS and centrifuge for 5 min at 200 x *g*, at RT.
- Subject the cell pellets to HBSS treatment with (**0.5, 1, 3, 10, 30,** and **100) µM** H_2DCFDA by gently pipetting up and down.
- Incubate the cells (resuspended in tubes) in a cell incubator [(37 °C), high relative humidity (95%), and controlled CO_2 level (5%) in the dark for 45 min.
- Following incubation, wash the cells with ice-cold PBS.
- Add **PI** (0.5 µl/0.5 ml/tube) (final concentration = 1 $\mu g \cdot ml^{-1}$) before placing the tubes on ice (still in the dark) and proceed immediately to flow cytometry inspection.
- For an easier follow-up, Fig. 8 summarizes the chronological steps.

Note the following:

1. The PI staining should be performed immediately before flow cytometer analysis.
2. To minimize cell activity, place the tubes on ice. This avoids artificial fluctuations in ROS detection between the first and subsequently analyzed tubes, in FACS.
3. It is documented that in flow cytometry a total of 5000 events are analyzed.
4. The cellular viability is examined via PI staining. Measure the PI fluorescence in the FL-3 channel (620 nm) of FACS Vantage SE (Becton Dickinson)/any other company.
5. The PI negative living cells were selected by FACS gating, in which, one could record the DCF fluorescence on the FL-1 channel (525 nm) of FACS.

7.3.2 Superoxide Measurement by Nitroblue Tetrazolium Method

- The reduction of nitroblue tetrazolium (**NBT**) to insoluble blue color formazan can be used as a probe for superoxide generation, although it is not entirely specific for superoxide.

- A designated hemocyte population (usually **5 x 10^5**, other numbers of cells can also be used) may be deposited in triplicate, in **100 µl** final medium volume in wells of a 96-well microtiter plate. Afterward, it is incubated in humid conditions for 30 min at RT to allow adherence of the hemocytes.
- The supernatants were then eliminated and replaced by a 50 µl medium, after which a 50 µl medium containing respiratory burst elicitors (or not), and with or without inhibitors, is added.
- Prepare a 0.3% NBT solution in the appropriate medium. Add 50 µl of this solution to the wells.
- Following 2-h incubation, the supernatants should be removed after which the hemocytes are fixed by adding 200 µl absolute methanol. This is followed by washing twice with 70% methanol and subsequent drying.
- Add 120 µl, 2 M KOH, and 140 µl DMSO to solubilize the deposited formazan.
- After homogenization of the well contents, the extinction was read at 620 nm in a spectrophotometer (Choi et al. 2006).

7.3.3 Screening the Reactive Oxygen Species by Chemiluminescent Probes

- A commonly used technique for $O_2^{\bullet -}$ quantification is **lucigenin-**enhanced chemiluminescence.
- Other widely used chemiluminescent probes include **luminol, MCLA,** and **coelenterazine**.
- The major limitation of luminol is the indiscriminate recognition of various free radicals. However, luminol is still in use to ascertain the $O_2^{\cdot -}$ prevalence in the presence of other free radicals.
- For the detection of superoxide free radicals, one of the most commonly used chemiluminescent probes is lucigenin (**bis-*N*-methylacridinium nitrate**), an **aromatic compound**. It exhibits **bluish-green** fluorescence.
- These methods are not specific to mitochondria.
- The validity of this technique has been questioned because $O_2^{\bullet -}$ production might be synthetically overestimated owing to an occurrence of **redox cycling**, wherein the lucigenin radical reacts with oxygen to generate $O_2^{\bullet -}$.
- However, validation studies inferred that auto-oxidation does not occur at (1–5) µM lucigenin concentrations (Li et al. 1998). For most cell types, a 5 µM extent is adequate and higher concentrations should not be used.
- In the case of induced conditions with purified flavin proteins and NADH, the lucigenin-derived redox cycling is significant.
- However, this problem may be overestimated in intact cells and tissues with null NADH/NADPH supplementation.
- The luminescent biomolecules and the methods associated with these molecules are gradually supplemented by the other methods.
- For detailed protocol, one can use the following reference (Tarpey et al. 1999).

7.3.4 Reactive Oxygen Species Measurement by Fluorescent Probes

- For cellular and mitochondrial ROS ($O_2^{\bullet -}$) detection, **dihydroethidium (DHE)** and its mitochondrion-targeted form **mitoSOX** are used.
- The DHE is freely permeable to the cell membrane and nonspecifically reacts with superoxide to form a red fluorescent product **(ethidium)**. It also specifically reacts with superoxide to form **2-hydroxyethidium**.
- Accurate determination of fluorescence spectra from 2-hydroxyethidium is not possible (using confocal microscopy or other fluorescence-based techniques) because its fluorescence spectra overlapped with that of ethidium's (Dikalov et al. 2007; Kalyanaraman et al. 2012).

Mitosox-Enabled Mitochondrial Reactive Oxygen Species Measurement

- Grow the cells in 96-well plates.
- Wash the cells with PBS or HBSS (phenol red-free).
- Incubate cells with (~2.5 to 5) μM mitoSOX in FBS or HBSS for about 30 min at 37 °C.
- Remove mitoSOX.
- Wash cells with PBS (or HBSS).
- Fix the cells in 4% paraformaldehyde at RT.
- If necessary, counter stain with a mitotracker or DAPI.
- Then plates are read in 96-well plate reader (Zielonka and Kalyanaraman 2010).

NB: Always make a fresh working dilution of mitoSOX as these dyes are extremely prone to self-oxidation. Methanol fixation should be avoided because it tends to open the mitochondrial pores and dissipate the stain into the cytoplasm.

7.3.5 Superoxide Detection by Ferricytochrome C

- Generally, ferricytochrome C detects large amounts of superoxide released by the cells.
- As a biochemical reaction, ferricytochrome C reacts with superoxide to form ferrocytochrome C (equation below).

$$\mathbf{Fe^{3+} - cytc + O_2^{\bullet -} \rightarrow Fe^{2+} - cytc + O_2}$$

- The reaction is followed by measuring the spectrophotometric absorbance at 550 nm.
- The absorbance at the neighboring wavelengths of 540 to 560 nm is used as isosbestic points to normalize the 550 nm signal because of the unchanged neighboring wavelengths.
- The reduction of ferricytochrome C is not implicitly superoxide specific, but other enzymes and molecules are also capable of doing so. Therefore, additional assay (such as the role of SOD) must be completed to determine the exact superoxide concentration.
- Cytochrome C reduction is suitable for quantifying $O_2^{\bullet -}$ released during the respiratory burst of neutrophils or by the isolated enzymes (Azzi et al. 1975).

Limitations of Using Ferricytochrome C

As discussed in the above paragraph, ferricytochrome C can react with other molecules such as **glutathione, xanthine oxidase**, and **ascorbate**. Therefore, the results may not be exclusive attributes to superoxide.

Unfortunately, peroxynitrite ($ONOO^-$) and H_2O_2 present in the cells reoxidize the reduced ferricytochrome C back to its prototype, creating a cyclic condition and measurement of inflated data. However, under this condition, the addition of inhibitors or scavengers (catalase for H_2O_2 or urate for $ONOO^-$) could bypass this reoxidation.

- In this method, it is difficult to detect the lower $O_2^{\bullet -}$ quantities generated by **nonphagocytic cells** such as vascular smooth muscle cells and endothelial cells.
- Ferricytochrome C, being a large protein, does not have an access to the intracellular space and therefore cannot be used to detect $O_2^{\bullet -}$, prevailing in the cytoplasm or mitochondria of intact cells.
- Finally, reactions of cytochrome C with H_2O_2 and various commonly used drugs such as **oxypurinol (xanthine oxidase inhibitor), apocynin (NADPH oxidase inhibitor), and L-NAME (NO synthesis inhibitor)** can attenuate its reduction and consequently interfere in accurate $O_2^{\bullet -}$ detection.
- To verify the specificity of this reaction, exogenous superoxide dismutase (**SOD**) is used. In this case, superoxide could be estimated indirectly, via cytochrome *c* reduction.
- SOD is also utilized to measure superoxide generated from NADPH oxidase. Additionally,
- acetylation or succinylation of ferricytochrome C could improve its superoxide specificity, resulting in a kinetically decreased reaction rate.

7.3.6 Immuno-Spin Trapping Aided Reactive Oxygen Species Measurement

- This is a highly sensitive method that greatly expands the utility of spin trapping.
- This method detects the **modified protein adducts**, but **not the free radicals such as $O_2^{\bullet -}$ or OH•. Thus, all the types of free radicals cannot be detected by this method**.
- **5,5-dimethyl-1-pyrroline-N-oxide (DMPO) is** utilized for the immuno-spin trapping method. This molecule reacts with protein-free radicals and forms epitope(s) that are identified via immunological methods.
- A panel of antibodies developed by *Mason and colleagues* reacted with **DMPO-protein adducts**. At present, **immunostaining, immunofluorescence, Western blot,** and **flow cytometry** are used to determine **DMPO-protein and DMPO-antibody interactions**.
- The mitochondrial electron transport chain components have been detected using the electrospun trapping method.
- In the beginning attempts, anti-DMPO enabled the detection of DMPO-protein myoglobin and hemoglobin adducts which were formed on account of H_2O_2-mediated self-peroxidation (Villamena and Zweier 2004; Griendling et al. 2016).

8 Measurement of Reactive Nitrogen Species

- In 1992, significant research on **nitric oxide (NO)** coined it the referral of **"Molecule of the Year." Physiologically NO acts as a secondary messenger**. Some of the biological functions of NO include regulation of endothelium, arterial vasodilation and blood pressure, contraction/relaxation of vascular smooth muscle cells and vascular tone, platelet activation, cytotoxicity, neuroplasticity, and inflammation. In 1998, the **Nobel Prize** in **Physiology and Medicine** was awarded to **Robert F. Furchgott, Louis J. Ignarro,** and **Ferid Murad** (American pharmacologists) for their work on NO as a cardiovascular signaling molecule.
- The diatomic molecule NO is considered a free radical (**π-radical**).
- The atomic structure of NO is characterized by 08 electrons of an oxygen atom and those of 7 for nitrogen. Therefore, the reactivity of NO is based on 01 unpaired electrons.
- However, NO is a **relatively stable** molecule because the unpaired electron is delocalized between nitrogen and oxygen and may be used by both atoms.
- The gas form of NO is stable in absence of oxygen.
- In the mammalian cell cellular system, three NO synthesizing enzymes are present, enabling its synthesis from various cells. The first of these enzymes is endothelial nitric oxide synthase (**eNOS**), expressed by endothelial cells and some other cells. The phagocytic cells such as macrophages express inducible nitric oxide synthetase **(iNOS)**, the second enzyme involved in NO synthesis. The neuronal cells express a separate NO synthetase called neuronal NOS or **nNOS (the third NO synthesizing enzyme)**. The nNOS, iNOS, and eNOS are also called **NOS-1, NOS-2, and NOS-3** respectively.
- In addition to all these three NOS, mitochondria also produce special NOS called **mitochondrial** NOS (**mtNOS**).
- While eNOS and nNOS are Ca^{+2} dependent, the iNOS is Ca^{+2} independent.
- **The NOS oxidizes L-arginine through molecular oxygen, producing one molecule of NO and another of L-citrulline in a 1:1 molar ratio**.
- **The cofactors needed for L-arginase actions are flavin adenine mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin, heme, and NADPH**.
- The biological effects of NO are modulated by transcriptional, posttranscriptional, and posttranslational modulations. For instance dimerization, S-nitrosation, phosphorylation, interaction with modulatory proteins, inhibition by endogenous methyl-arginines, etc.
- Several factors are identified that are responsible for reduced NO generation and NOS uncoupling mediated superoxide production.

The most important factors are as follows:

The insufficient availability of L-arginine.
The insufficient availability of cofactors such as tetrahydrobiopterin.
S-glutathionylation of certain NOS isoforms.

In presence of superoxide, NO immediately (within a few seconds) reacts with it and generates several molecules. One of the most important reactive nitrogen species (**RNS**) produced in this direction is **peroxynitrite**, and another molecule that is formed by reacting with thiol molecules is **nitrosothoils (RSNO)**. The reaction of peroxynitrite with other molecules forms **nitrogen dioxide ($\bullet NO_2$) and dinitrogen trioxide (N_2O_3)** and **other free radicals**.

The RNS causes damage and stressful situations in the cells, together termed **nitrosative stress**. **The ROS and RNS, collectively cause huge damage to the mammalian cellular system**.

The peroxynitrite interacts with various cellular molecules including amino acids, DNA bases, lipids thiols, and low molecular weight antioxidants, at a relatively slow rate.

Peroxynitrite gets the chance to selectively react with various molecules since the reaction rate is slow.

Some of the important RNS-dependent reactions are as:

$$ONOO^- + H^+ \rightarrow ONOOH(\text{peroxynitrous acid}) \rightarrow \bullet NO_2(\text{nitrogen dioxide}) + \bullet OH(\text{hydroxyl radical})$$

$$ONOO^- + CO_2(\text{carbon dioxide}) \rightarrow ONOOCO_2^-(\text{nitrosoperoxycarbonate})$$

$$ONOOCO_2^- \rightarrow \bullet NO_2(\text{nitrogen dioxide}) + O = C(O\bullet)O^-(\text{carbonate radical})$$

$$\cdot NO + \cdot NO_2 \rightleftharpoons N_2O_3(\text{dinitrogen trioxide})$$

- Proteins such as hemoglobin, myoglobin, etc. may contain transition metals at the center. Peroxynitrite may react with the ferrous moiety of the hemoglobin/myoglobin and oxidize these proteins to their corresponding ferric form.
- Additionally, various amino acids of the proteins may react with peroxynitrite. For example, **cysteine oxidation** and **tyrosine nitration** are mediated by peroxynitrite.
- The reaction of peroxynitrite with amino acids and proteins leads to structural-functional alterations of various proteins including those of the cytoskeleton. This may severely damage several cell signaling pathways (Jourd'heuil et al. 2001; Wardman 2008).

8.1 Nitric Oxide Measurement Techniques

- Both **direct** and **indirect methods** are used for the NO measurement. However, indirect methods have dominated the field of NO measurement.

  The indirect methods of NO measurement involve the following:

  Determination of NOS Activities.
  Determination of the Activation of No Molecular Targets.
  Determination of the No Products.

NB: Generally, GC-derived cGMP is measured for quantifying the NO molecular targets. Similarly, for the amount of NO reaction products, the relative proportion of RSNOs or nitrite/nitrate is used.

- Various **spectroscopic** and **electrochemical** methods utilized to measure NO include **colorimetry, fluorescence quantification, luminescence assessment,** and **electron spin resonance spectroscopy (ESR)**.
- The accuracy and exactness of these methods for NO quantification vary with each other and are relatively unable to make an in situ assessment of NO expression within the physiological boundaries.
- The direct quantification assays are rather more specific, substantially due to the less common practice of screening the ESR after the in vivo or ex vivo spin detainment or the explicit NO-specific biosensors (Green et al. 1982; Granger et al. 1999; Csonka et al. 2015).

8.2 Nitric Oxide Measurement by Griess Method

8.2.1 The Griess Reaction

- The **Griess** test is an analytical chemistry method for detecting nitrate ions in solution. One of the most important uses of this test is the determination of nitrite in drinking water. The **Griess** diazotization **reaction** on which the **Griess** reagent relies was first described in 1858 by *Peter **Griess***.
- A two-step method is used to measure the nitrate (NO_3^-) and nitrite (NO_2^-) combined extent within the samples. In the first of these, NO_3^- is initially transformed to NO_2^-, using enzymatic assisted reduction of metallic cadmium, succeeded by the estimation of total NO_3^- in the sample being examined, using stoichiometric diazotization. This reaction involves a Griess reagent to generate a purple azo product. The native NO_3^- extent can be computed for samples for which NO_3^- has remained unconverted, thereby allowing the NO_3^- quantification via difference calculation.
- Of the two enzyme-mediated conversions known for NO_3^- to NO_2^- conversion, one used the nicotinamide adenine dinucleotide phosphate (NADPH) dependent nitrate reductase. This enzyme is obtained from *Aspergillus* species and is quite robustly available on an industrial scale. The second enzyme is formate nitrate reductase obtained from *Escherichia coli*.
- These methods have been well accustomed for the NO_3^- conversion and NO_2^- quantification in a 96-well plate with no need for sample transfer, a benefit they hold over the cadmium-mediated conversion.
- Besides, these methods eliminate the hurdle of exposing the laboratory personnel to the deadly toxic Cd and ease the challenges for discarded wastes (Sun et al. 2003).

8.2.2 NADPH-Based Nitrate Reductase

- The first description of simultaneous NO_3^- and NO_2^- quantification with the aid of NADPH-based nitrate reductase, was provided by *Wu and Brosnan*. The

method works on NADPH-driven nitrate to nitrite reduction accompanied by the quantification of total NO_2^- formed using Griess reagent.

- This hypothesis was gradually improved by Verdon and accomplices, by including the glucose-6-phosphate (G-6-P) or G-6-P dehydrogenase NADPH reformation loop. The modification averted the interference observed during the color development in the Griess reaction caused by the high $NADP^+$ titer.

$$NO_3^- + NADPH + H^+ \xrightarrow{\text{Nitrate reductase}} NO_2^- + NADP^+ + H_2O$$
$$G\text{-}6\text{-}P + NADP^+ + H_2O \xrightarrow{G\text{-}6\text{-}PDH} 6\text{-}PG + NADPH + H^+$$

8.2.3 Formate-Nitrate Reductase

The NO_3^- to NO_2^- transformation via involvement of formate-nitrate reductase relies on the method proposed by *Taniguchi and associates*. In this method, flavin mononucleotide (FMN) is included as a supplementary electron carrier which is reduced via formate dehydrogenase action (formate implies ferricytochrome b_1 oxidoreductase) of this complex to $FMNH_2$. This $FMNH_2$ is thereafter used to catalyze NO_3^- to NO_2^- reduction using the nitrate reductase action (ferrocytochrome infers NO_3^- oxidoreductase). Contrary to NADPH and $NADP^+$, the FMN does not cross-interpreted with the Griess reaction.

The NO_2^- generated in the reaction is quantified using the Griess reagent.

$$FMN + H_2O + HCOO^- \xrightarrow{\text{Formate dehydrogenase}} CO_2 + OH^- + FMNH_2$$
$$FMNH_2 + NO_3^- \xrightarrow{\text{Nitrate reductase}} NO_2^- + FMN + H_2O$$

8.2.4 Materials

Griess Solution A

Dissolve 0.1 g N-(1-naphthyl) ethylenediamine hydrochloride in 100 ml double-distilled water. Store at (0 to 4) °C in a light-protected environment.

Griess Solution B

Dissolve 1 g sulfanilamide in 100 ml, 5% (v/v) orthophosphoric acid. Store this too at (0 to 4) °C in a light-protected environment.

Working Griess Reagent

Prepare the working Griess reagent by mixing stoichiometrically equal contents of Griess solution A with Griess solution B. Make the mixture immediately before use.

Nitrate Standard (10 mM)

Dissolve 85.0 mg sodium nitrate in 100 ml of double-distilled water. Store at (0 to 4) °C in a light-protected environment. Prepare standard solutions from this stock ranging between (0 to 100) μM NO_3^-.

Nitrite Standard (10 mM)

Dissolve 69.0 mg sodium nitrate in 100 ml double-distilled water. Store at (0 to 4) °C in a light-protected environment. Prepare standard solutions from this stock ranging between (0 to 100) μM NO_2^-.

NB: Do not use phenol red-containing medium as phenol red can interfere with the color determination in the Griess reagent.

Nitrate to Nitrite Conversion Using the Formate Nitrate Reductase Assay

0.5 M Phosphate Buffer, 0.8 M Sodium Formate, 0.4 mM FMN Buffer, pH 6.0

Dissolve 0.61 g $Na_2HPO_4 \cdot 2H_2O$, 3.36 g $NaH_2PO_4 \cdot 2H_2O$, 2.72 g sodium formate (Sigma F 6502), and 10 mg FMN (FMN-Na·$2H_2O$, Sigma F 8399) in 50 ml double-distilled water. Adjust the pH to 6.0 and store at (0–4) °C in a light-protected environment.

Preparation of Reagents for the NADPH-Dependent Nitrate-Reductase Assay

Sodium Phosphate Buffer, PH 7.4 (14 M)

Dissolve 101 mg $Na_2HPO_4 \cdot 2H_2O$ and 20.8 mg $NaH_2PO_4 \cdot 2H_2O$ in 50 ml double-distilled water. Store at (0 to 4) °C.

Nicotinamide Adenine Dinucleotide, Reduced Form (10 M)

Dissolve 1.7 mg NADPH (Sigma N 6504) in 2 ml double-distilled water. Dilute 100-fold to obtain the working solution. Prepare fresh before use.

Conversion Buffer Sufficient for 125 Samples

Dissolve 3.8 mg glucose-6-phosphate in a 14 mM phosphate buffer. Add two units of glucose-6-phosphate dehydrogenase (final concentration 400 $U \cdot l^{-1}$) and one unit of NADPH-dependent nitrate reductase (from *Aspergillus* species, 200 $U \cdot l^{-1}$ final concentration). Prepare fresh before use.

Preparation of Reagents for Converting Formate-Nitrate Reductase (Approx 2–4 KU/L).

Suspend the lyophilized powder to a final concentration of 2 mg protein per ml in double-distilled water at 4 °C. Prepare immediately before use, from lyophilized powder.

8.2.5 Methods

Nitrite Estimation in Test Samples

- Transfer 100 μl sample or nitrite standard in a 96-well microtiter plate using a pipette. In case, the sample volume is less than 100 μl, the volume should be made up to 100 μl using water or the medium that was used for preparing samples.

- Supplement 100 μl Griess working solution before incubating at RT for 15 min. This results in the formation of color.
- Determine the absorbances of samples and standards at 540 nm.
- Estimate the NO_2^- titer of the samples by comparing them with the nitrite standard curve.
- In the case of mere NO_2^- determination, a standard curve is linear till 250 μM.

Estimation of Total Nitrate and Nitrite Amounts through NADPH-Dependent Nitrate Reductase Assay

- Transfer 50 μl sample and NO_3^- or NO_2^- standards to a 96-well microtiter plate.
- Add 40 μl conversion buffer to each well.
- For quantifying the NO_2^- alone, make a conversion assay buffer, not contain NADPH-dependent nitrate reductase. Pipette out 40 μl of this in a similar set of wells having the samples.
- Add 10 μl of NADPH solution to each well, using a pipette.
- Shake the plate thoroughly before 45 min of incubation at RT. This converts all NO_3^- to NO_2^-.
- Use the Griess reagent to estimate the total NO_2^- content in the samples. Contrast the absorbances for NO_3^- and NO_2^- standard curves.
- For the NO_3^- standards ranging within (0–100) μM NO_3^-, the net NO_3^- to NO_2^- transformation is >95%.
- Estimate the gross NO_2^- concentration in the samples using the standard curves.
- This exercise provides the stoichiometrically transformed NO_2^- from NO_3^- including the NO_2^- already prevailing in the medium.
- Calculate the NO_3^- titer by subtracting the NO_2^- concentration determined without enzyme, from the total NO_2^- content. If needed, correct this estimate so that it facilitates the estimate of percentage transformation.

Formate Nitrate Reductase Assay for Total Nitrate and Nitrite Determination

- Dilute one portion of phosphate conversion buffer with 1.8 proportion of double distilled water, before use.
- Transfer 50 μl of sample NO_2^- and NO_3^- standards into the wells of a 96-well microtiter plate using a pipette. Add 40 μl conversion buffer along with 10 μl diluted formate nitrate reductase.
- The NO_2^- contents of samples could be determined via adding conversion buffer supplemented with 10 μl water, compensating the enzyme. In the absence of an enzyme, the NO_3^- conversion cannot happen.
- Incubate at 37 °C for 60 min for NO_3^- to NO_2^- transformation.
- Estimate the total NO_3^- concentration in the samples using the Griess reagent. Now determine the NO_2^- titer in the original medium.

Procedure for Formate-Nitrate Reductase Preparation

- Grow *E. coli* (strain JCB387), *pcn*Δ*nrf* via anaerobic mode, using a 5 ml culture medium having 10 g LB medium supplemented with 5 μl priorly prepared (as stock) kanamycin solution till 8 h.

- Aseptically relocate 0.5 ml culture to 500 ml LB medium. Allow it to grow overnight.
- Harvest the cells using centrifugation at 2000 x *g* for 20 min, at 4 °C.
- Resuspend the cells in 500 ml distilled water and wash the cells via centrifugation at 2000 x *g* till 20 min at 4 °C. This removes all sorts of NO_3^- or NO_2^- from the medium.
- Repeat the washing step at least twice.
- Freeze the cell pellet for (2–16) hours at -20 °C, to eliminate any sort of formate-nitrite reductase.
- Resuspend the cell pellet (~ 2 g) in 10 ml (5 volumes) of ice-cold distilled water. Disrupt the cells by sonicating for 5 min followed by cooling ((10–20) kilocycles).
- Centrifuge the sonicated cell mass at 2000 x *g* for 20 min at 4 °C. This enables the retrieval of nondamaged cells.
- Discard the supernatant and centrifuge again at 2000 x *g* for 40 min to collect the membrane fraction having membrane-bound formate-nitrate reductase.
- Wash the pellet twice via resuspending in distilled water. Thereafter, again conduct centrifugation at 2000 x *g* for 40 min at 4 °C.
- The cell pellet retrieved in step 8 could be resuspended in distilled water. It can be further sonicated and centrifuged (steps 7–9), to enhance the yield as desired.
- The net membrane fraction is resuspended in 1 ml distilled water before dialysis with 1 liter distilled water at 4 °C overnight.
- Optimize the protein load of dialyzed sample to 10 $mg \cdot ml^{-1}$.
- This sample could be used as such in the nitrate-reductase assay. For long-term storage, lyophilize small aliquots and store them at -70 °C.
- For an easier follow-up, kindly refer to Fig. 9.

Cautions:

- Strains as variable as, Yamaguchi (IFO 12433), strain B, or amicable *E. coli* cultures could be used, subject to their adequate possession of formate nitrate reductase. While using these, prefer not to add kanamycin. Normal *E. coli* strains have a certain amount of formate nitrate reductase (FNR). It is inevitable to prevent the FNR loss in course of freezing and extensive membrane washing. The repeated interference caused by the residual traces could be ascertained by comparing the NO_2^- standard curves, constructed with and without enzyme involvement after incubation.
- A range of compounds may prevail within the sample that interfere with NO_2^- diazotization and ultimately with Griess reaction. Such compounds could be reducing agents like cysteine, glutathione, ascorbic acid, NADH, or NADPH.
- Screening of such interfering compounds in the various samples could be made *by* adding known NO_2^- extents, as internalized standards.
- In case the constituents of the Griess solution are included separately, an inadvertently greater absorbance could be obtained. It is therefore much preferred to include the premixed Griess reagent while processing a large number of samples.

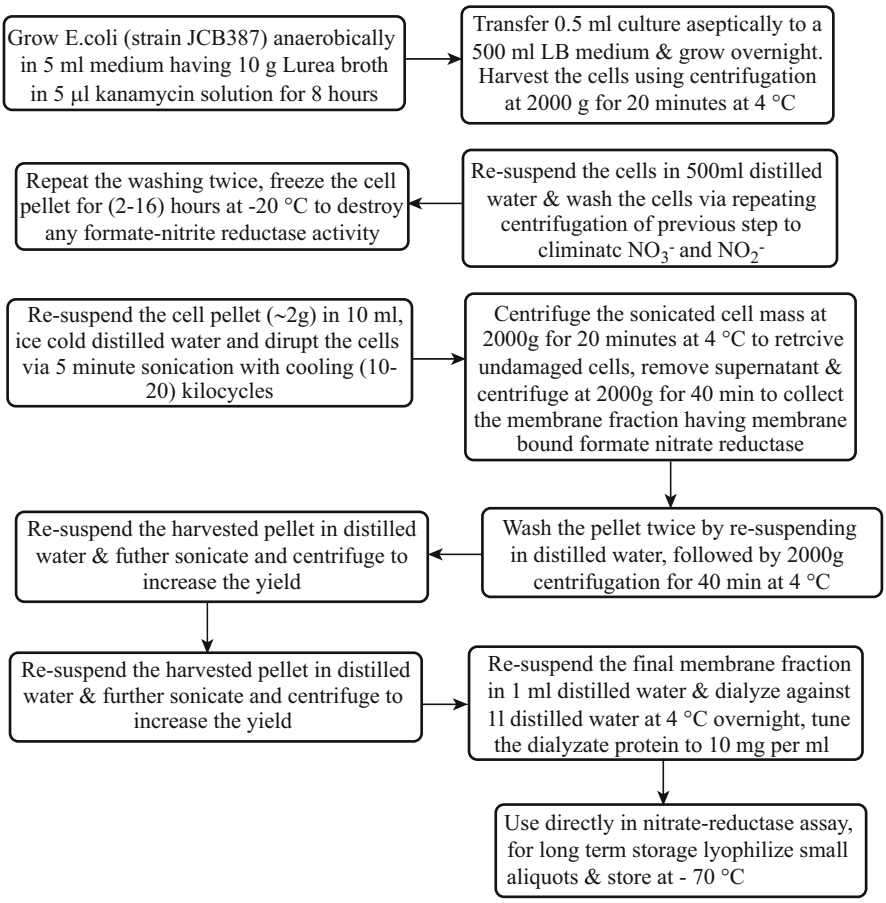


Fig. 9 Sequential steps for the preparation of formate nitrate reductase

- In the event of the requirement of higher sensitivity, 50 µl N-(1-naphthyl)ethylene diamide hydrochloride solution should be supplemented. To expedite the color development, this is succeeded by a prompt inclusion of a 50 µl sulfanilamide solution.
- The primary filters with a typical range of (530–570) nm could very much suit the assay requirements.
- Samples should be preserved at -20 °C and must be promptly analyzed.
- To get rid of any undesired NO_3^- and NO_2^- metabolism (caused by accidental bacterial or mold contamination), the samples should be immediately sterilized via filtration. In general, such assays do not mandate protein precipitation. However, if it is a dire necessity, the method of precipitation must be carefully selected to avoid any infecting interference of nitrate reductase enzymes.

8.2.6 Urine Samples

In general, the NO_3^- and NO_2^- concentration in the urine samples remains within (200–2000) μM, owing to which, dilution by a factor of ~10 is needed. For formate-nitrate assay (FNR), this dilution is made in the water while for NADPH-dependent nitrate reductase assay, it is made using assay buffer. Usually, dilutions to an extent of (0.5–10)% (v/v) do not alter the conversion in either of these assays. However, optimization of incubation time may be crucial in course of NADPH-dependent nitrate reductase assay, to accomplish a maximum conversion.

8.2.7 Culture Media

Various tissue culture media generally carry a high NO_3^- concentration, resulting in their subsidual via those having moderate NO_3^- quantities. Certain media configurations may interrupt the color formation (might not always be the conversion), and exhibit minor deviations from the linearity during the Griess reaction, in particular for those having high serum content. This can be resolved via using the standard dilutions of NO_3^- and NO_2^- stock culture media and using curve-fitting algorithms instead of standardized linear regression to compute the unknown extents using standard plots. Further, it may not be 100% necessary to enhance the incubation time (for maximal conversion) using the NADPH-dependent nitrate reductase assay. Alternatively, the exact time for conversion for each sample configuration must be recorded.

8.2.8 Plasma Samples

Quantification of NO_3^- and NO_2^- can be made without any loss to the protein content of the plasma membrane. It is always preferred to dilute the plasma (to 20%), either with water for the FNR assay or assay buffer for the NADPH-dependent nitrate reductase assay. In case of substantial particulate matter prevalence in the plasma, it should be priorly centrifuged or filtered to eliminate any undesired interference. The diluted plasma samples generally, do not modulate the relative conversion extent, though it does interrupt the NO_3^- estimation amidst the Griess reaction, reducing the maximum absorbance by ~10%. This can be resolved *by* comparing the standards made in water and diluted serum. In the case of <95% NO_3^- to NO_2^- transformation, the incubation period should be increased via the involvement of converting enzyme.

- The NADPH should be essentially prepared separately for the reaction to proceed.
- If assay sensitivity is an issue, initial dilution of conversion buffer could be bypassed, increasing the sample volume to 75 μl. Thereafter, 15 μl undiluted conversion buffer is used for supplementation.
- While screening multiple samples, the enzyme could be added to the conversion buffer just before use. In such an event, 50 μl of this mix is refurbished to each well. Herein, it needs to be ensured that the enzyme prevails in a suspension state.

8.2.9 Measurement

The solutions required for quantification are sulfanilamide, N-1-naphthyl ethylenediamine dihydrochloride (NED), and NO_2^- standard. The developed assay is termed as Griess Assay.

NED Solution

Make a 0.1% N-1-naphthyl ethylenediamine dihydrochloride solution diluted in sterile water. Sulfanilamide solution: Make a 1% sulfanilamide solution, diluted in 5% phosphoric acid.

Nitrite Standard

Dilute the 0.1 M standard stock sodium nitrite to 100 μM in a sterile medium, and do a serial dilution in the same medium.

Storage Conditions

Chemicals should be stored as directed by the manufacturer at room temperature (RT). When reconstituted, NED and sulfanilamide solutions are stored immediately after use at 4 °C, in the dark, for a maximum of 3 months.

Cell Culture

Culture cells (**RAW 264.7, a mouse macrophage cell line**) in a 96-well plate, in the triplicate mode for each condition. Caution must be exercised to include proper controls according to experimental specifications.

8.2.10 Cell Treatment

- Treat cells to induce NO_2 production. One can use lipopolysaccharide (LPS) (100 $ng \cdot ml^{-1}$) and recombinant IL-4 to treat the cells.
- Both reagents must be made amenable at RT to commence the assay.
- Spin the culture plates and retrieve the respective supernatants to facilitate a 50 μl culture transfer over to a new 96-well plate. The dilution wells of the standard stock are optimized or make a standard curve.
- Supplement each sample and control with 50 μl sulfanilamide solution. Mix well.
- Incubate in dark at RT for 10 min.
- Supplement all samples as well as control with 50 μl N-1-naphthyl ethylenediamine dihydric chloride solution and mix well.
- Yet again, incubate at RT in dark for 10 min.
- Record the absorbance using a plate reader equipped with (520–550) nm wavelength filter.
- In the vent of varying sized plates and dishes being used, prefer mixing 1:1 proportions (volumetric) of each solution and sample supernatant.
- Violet color is formed in the positive wells. The observations of standard formulation would be of help in predicting the solution stability.

NB: After treating the cells with the desired factor, proceed with cell harvest and collect both pellet and supernatant. The supernatant should be tested using NO

assay while the pellet should be screened for iNOS expression via *Western Blot. This is the right stage to correlate both results*.

8.2.11 Measurement of Peroxynitrite

- For peroxynitrite ($ONOO^{\bullet -}$) detection, **dihydroergotamine (DHR)** is commonly used.
- In this assay, two electrons oxidized product rhodamine is produced by the $ONOO^{\bullet -}$-dependent oxidative conversion of DHR.
- Since DHR is also reduced by thiols and ascorbic acids, it may give false results.
- At present, DHR is used as a nonspecific indicator of intracellular $ONOO^{\bullet -}$ and HOCl or other one-electron oxidants (Martin-Romero et al. 2004).

9 Conclusions

This chapter used in vitro cultured mammalian cells to provide a step-by-step description of various assays related to multiple physiological and pathological events. Initially, there were multiple assays related to the determination of cell survival and proliferation such as membrane integrity inspection, dye exclusion determination, trypan blue exclusion method, dye uptake efficacy, labeled chromium uptake, clonogenic assay, enzyme release assay, assays based on radioisotope incorporation, colorimetric detection, and cellular respiration. Then various assays related to cell adhesion and migration such as in vitro scratch assay and Boyden Chamber assay are discussed. The chapter also focused on various assays related to program and nonprogrammed cell death such as caspase activation and detection, cytochrome C release assay, DNA condensation and fragmentation, and Annexin V apoptosis assay. Finally, various assays and techniques related to the measurement of ROS and RNS such as the spectroscopic method, fluorescent method, chemiluminescent probes, spectrophotometric, chromatographic and electrochemical sensing (for ROS) and Griess reaction, NADPH-dependent nitrate reductase assay, formate nitrate reductase assay, and peroxynitrite measurement (for RNS) were included. A thorough understanding of all the above-mentioned techniques is highly essential for the basic research on cell biology and cell physiology as well as for industrial purposes to screen various drugs/toxins against multiple disorders like cancers, generation of recombinant proteins, and vaccines against various viruses, and monoclonal antibody production.

10 Cross-References

► Isolation and Purification of Various Mammalian Cells: Single Cell Isolation

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Mammalian Cells, Tissues and Organ Culture: Applications

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_17-2

Abstract

In today's world, mammalian cell cultures are used to understand various physiological and pathophysiological cell signaling events related to normal as well as various diseased cells such as cancer cells and others. Besides, mammalian cells such as Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, human embryonic kidney (HEK) cells, African green monkey kidney (COS) cells, NSO cells, HT1080 cell, and PER-C6 cells (many others too) as well as their culture products (various recombinant proteins) have widespread use in biotechnology, pharmacology, and medicine. The major usefulness of cultured mammalian cells and their products described in this chapter include (i) use as a model system for physiological and pathophysiological studies; (ii) use in experimental drug/toxin research; (iii) use in vaccine production, particularly against pathogenic viruses; (iv) use for various recombinant protein production; (v) in cell therapy; and finally (vi) in gene therapy. The various mammalian cell culture recombinant DNA products described in this chapter are tissue plasminogen activator, urokinase, follicle-stimulating hormone, blood clotting factor VIII, and erythropoietin. All these recombinant proteins exhibit widespread significance in the medical field. In comparison, cell and gene therapy are new fields and their success against various human diseases is substantially in the initial stages.

Keywords

Chinese Hamster Ovary (CHO) cells · Baby Hamster Kidney (BHK) cells · Human Embryonic Kidney (HEK) cells · African Green Monkey Kidney (COS) cells · NSO cells · HT1080 cells · PER-C6 cells · Model for Physiological & Pathophysiological studies · Cell Culture Products · Recombinant Proteins · Vaccines · Cell Therapy · Gene Therapy

1 Introduction

The culture of mammalian cells has manifold significance in every sphere of modern-day life. Various mammalian cell lines such as Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, human embryonic kidney (HEK) cells, African green monkey kidney (COS) cells, NSO cells, HT1080 cells, PER-C6 cells, etc. are widely used, not only for understanding the mammalian cell physiology but also for clinical, pharmaceutical, and industrial purposes. The shape, size, morphology, structure, and functions of the mammalian cells are examined using in vitro cultured mammalian cells. In vitro cultured mammalian cells are used to understand the physiology and pathophysiology of cell proliferation (growth), survival, and apoptosis. Of note, cell proliferation and programmed death (apoptosis) are prominent physiological phenomena of mammalian cells. The efficacy of various newly synthesized/discovered drugs/chemicals/toxins is examined using in vitro cultured mammalian cells. The drug discovery laboratories throughout the world examine the effects of newly synthesized anticancer drugs or drugs produced against other diseases amidst the various cell cycle phases (***G1-S-G2-M***) using cultured cancer cells. The positive results from the in vitro cell culture experiments can serve as key cautions and guidelines for in vivo animal culture and other experiments. The pathogenic animal viruses use animal cells for their multiplication and propagation. One of the most imperative benefits of mammalian cells is for studying viral propagation and generating antivirus vaccines. Perhaps the most important application of cultured mammalian cells is the production of several recombinant proteins that are immensely useful in pharmaceutical or biotechnological industries and clinical or medical sciences. Various steps involved in recombinant protein production in cultured mammalian cells comprise isolation, characterization, and optimization of the gene of interest, choosing a suitable vector, selection of a suitable mammalian host cell, DNA delivery and integration into the mammalian host cell, transient versus stable transfection, selection and optimization of a positive clone and mammalian expression system for mass culture, selection and optimization of cell culture medium for mass culture, selection and optimization of cultivation mode – adherent versus non-adherent (perfusion and fed-batch) – purification of the expressed or secreted proteins (from lysed cells or the culture medium), and finally quality control and quality assurances. The most important recombinant proteins produced using mammalian cells are tissue plasminogen activator (tPA, used to dissolve blood clots), urokinase (used to dissolve blood clots), erythropoietin (EPO, stimulates RBC production), follicle-stimulating hormone (FSH, used for infertility treatment), blood coagulation factor VIII (used for hemophilia A treatment), and various monoclonal antibodies (used against various pathophysiological or diseased conditions including cancers, rheumatoid arthritis, osteoporosis, sepsis, Crohn's disease, etc.). Additionally, mammalian cell culture is also used in replacement therapy (for replacement of old, damaged, or injured tissues and organs such as the skin), gene and cell therapy (for correcting genetic mutation), and cell fusion technology (such as hybridomas for the production of monoclonal antibodies). In a nutshell, the cultured mammalian cells have multiple significance,

in experimental research, physiology, and pathophysiology, and the relevant applications in biotechnological, pharmaceutical, and medical sciences. The present chapter describes all these utility aspects of mammalian cell culture.

2 Use of Mammalian Cells as a Model System for Physiological and Pathophysiological Studies

More than 200 years have passed since the father of modern experimental physiology, *Claude Bernard*, was born. Extensive research works of numerous scientists led to the complete present-day knowledge of mammalian physiology. The use of mammalian cells and the in vitro culture of these cells is one of the most important tools and techniques, respectively, for key discoveries in mammalian physiology and pathophysiology.

In vitro cell culture is frequently used to advance understanding of the mechanisms that underlie the in vivo cell behavior. These behaviors include cell growth (division/proliferation), differentiation, migration, and mechanics, all of which are sensitively influenced by their biochemical and biomechanical microenvironments. Deciphering the mechanisms behind these behaviors is vital to understanding the in vivo processes that eventually form the tissues and organs as well as allocating their functions. Ideally, laboratory experiments could be performed with a user-defined three-dimensional (3D) model that closely mimics the cellular microenvironment.

In vitro mammalian cell culture has the following significance:

- To understand the specific phenotype including shape and size (morphology) of a particular cell type (e.g., endothelial cells have a cobblestone morphology) both in 2D and 3D culture.
- To understand karyotyping and related genetic analysis of cells.
- To understand the effects of specific environmental conditions (e.g., temperature, pH, moisture, O_2/CO_2 tension, medium composition, nutrition, etc.) on the growth and physiological behavior of cells.
- To understand the processes through which cell division and proliferation take place along with studying the effects of varying nutrients, medium pH, temperature, moisture, etc. on the mammalian cells using cultured mammalian cells.
- For deciphering the role of various cyclins (cyclins D, E, A, and B) and cyclin-dependent kinases (CDKs: CDK4, CDK6, CDK2, and CDK1) in the cell cycle regulation (various phases: (G1-S-G2-M)). Experimental studies on cultured mammalian cells help to dissect the altered cell cycle regulation in non-transformed along with its implicit comparative progress in transformed cells.
- To understand the signaling processes involved in cell differentiation amidst 3D cell culture.
- To understand the basic physiological functions of a cell. For example, the mechanism through which a neuron sends nerve impulse (through patch-clamp

technique), a skeletal muscle contracts (sliding filament theory), or glucose transport by endothelial cells (via glucose transporter or GLUT), etc.
- To understand the cellular metabolism in real-time and metabolic distinctions of non-transformed and transformed cells (the Warburg effect).
- To understand the programmed (apoptosis) and non-programmed (necrosis) death of cells, from physiological and pathophysiological viewpoints.
- To understand the cell-to-cell (cross-talk) and cell-to-extracellular matrix (ECM), interactions, and possible association for directional signaling using integrins, examined using mammalian cell culture.
- Understanding the interaction between the cells and pathogens (host-pathogen interaction) can be demonstrated using cell culture.
- To understand the physiological defense mechanism.
- To understand the process through which various ions (Na^+, K^+, and Cl^-) and nutrients (e.g., glucose, amino acids, etc.) are transported across biological membrane channels.
- Observing the effects of various drugs, toxins, and other molecules on cultured mammalian cells are immensely useful.
- To use the mammalian cells for the transfection of viruses and producing antiviral vaccines.
- To use the mammalian cells for producing recombinant proteins, cytokines, chemokines, etc. Cultured mammalian cells are prime requirements for this.
- To understand the alteration of various cellular processes amidst diverse patho-physiological conditions (e.g., cancers) (Mills and Estes 2016).

Overleaf paragraphs (step-by-step) describe the prominent utilities of mammalian cell culture:

3 Use of Mammalian Cells for Experimental Drug/Toxin Research

The research and **development (*R&D*)** section of biotech/pharmaceutical companies or basic scientific research laboratories is primarily focused on **new drug discovery**. To observe the efficacy of a newly synthesized drug, the first step is to examine the effects of a drug on the in vitro cultured mammalian cells. Various mammalian cultured cells are widely used either alone or in conjunction with animal cells, to study the effects of new drugs, cosmetics, and chemicals on their survival, growth, and biological activities. For the treatment of cells with various drugs/toxins and other molecules, it is pertinent that cells in the culture container reach a particular confluence status. In two-dimensional cultures, confluency implies the area of the cell culture container covered by growing cells. For example, 100% confluence indicates the cell culture vessel is full/occupied by growing cells and there is no place left to grow. Depending upon the type of experiments, cells are grown to varying confluence extents followed by their treatment with different experimental agents. For example, for transfection assay, cells can be grown at

40–50% confluence, while for apoptosis assay, cells can be grown at 90–100% confluence. Standardization of the drug/toxin dosage or concentration alongside the treatment stage is highly essential to monitor the effects of any drugs/toxins/other agents (Ekwall 1980; Paganuzzi-Stammati et al. 1981; Ekwall 1983).

The following paragraphs describe the dose and time response of an experimental drug:

3.1 Dose-Response of the Experimental Molecules

Dose-response comprises the monitoring of various concentrations of a drug/toxin/another agent on the cultured mammalian cell growth for a fixed time, following treatment to determine the optimum effective drug concentration manifesting desirous experimental effects. For example, if a drug administered is intended for breast cancer treatment (curtailing the corresponding cell proliferation), dose-response will check the suitable drug concentration for inhibiting breast cancer cell proliferation following a fixed duration treatment. In dose-response monitoring, time of the time-lapse of treatment efficacy monitoring and delivered drug concentration need to be the same in all simultaneous analyses.

Briefly, the technical steps may be as follows:

- Take at least five (five) cell culture containers (plate/flasks) of the same type and size (like each as a 100 mm Petri plate).
- To each container, add the same number of cells with the same medium volume. Suppose $1x10^6$ endothelial cells are added to a 100 mm Petri plate with 10 ml M199 medium before being grown in a CO_2 incubator for up to 80% confluency. Other culture plates should ideally be a replica of this culture plate.

NB: The volume of the medium, as well as cell number, varies with different container sizes.

- Allow the cells to grow up to the desired confluence before drug treatment.
- Dissolve the experimental drug in a suitable solvent (water/organic solvent like ethanol/DMSO, etc.) depending upon the solubility of the drug/chemical.
- Serially dilute the chemical/drug with the solvent.
- Keep one cell culture vessel as control. To this control cell culture container, add only the vehicle/solvent in which the experimental drug has been dissolved and serially diluted.
- Add the drugs/chemical at logarithmic higher concentration (from lower to higher extent) using each cell culture container.
- For example, the 05 cell culture containers mentioned above can be used with the following drug concentrations:
  Plate 1: Solvent/vehicle alone (control)
  Plate 2: Added with 10 pM drug concentration
  Plate 3: Added with 30 pM drug concentration

Plate 4: Added with 100 pM drug concentration
Plate 5: Added with 1 nM drug concentration

NB: Depending upon the experiment, more plates with a logarithmic higher concentration of drugs may be needed. The concentrations acronyms signify the following: pM = picomolar, nM = nanomolar, μM = micromolar, mM = millimolar, M = molar.

- Following administration of the drug(s), all cell culture containers are incubated at 37 °C in a CO_2 incubator for the same time (say, for instance, 4 h).
- The treated cells may be utilized for various assays (e.g., MTT assay for cell proliferation) or lysed to isolate DNA/RNA/proteins before being used in a defined experiment. It should also be screened whether the experimental desired gene/mRNA/protein is up- or downregulated.
- Dose-response helps to determine the effective dose/concentration range of a drug corresponding to which the expected result/treatment responses are observed.

3.2 Time Response of the Experimental Molecules

Time response is the same as dose-response; the only exception is the fixed (experimental) drug concentration in all culture containers. However, the time of treatment varies from one to another cell culture container.

Briefly, the technical steps may be as follows:

- Following the dose-response of a particular drug/chemical, the most **effective** drug concentration is examined by the researcher.
- To determine the exact time at which the experimental drug is most effective, several similar-sized cell culture containers have to be grown as mentioned in the dose-response. The number of the cells and the volume of the same cell culture medium must be the same in all culture containers.
- All the containers except control (the ones treated only with vehicle/solvent) are treated with the same drug(s) concentration (the most effective drug concentration can be chosen from the dose-response) for varying periods.
- This means only one particular concentration (suppose 10 nM) of a drug could be added to every container for varying durations (e.g., control, 30 min, 1 h, 3 h, 10 h, etc.).
- From the time response, one can choose the desired time till when the maximum up- or downregulation of desired DNA/RNA/proteins takes place.
- The treated cells could be utilized for various purposes including multiple assays as mentioned in the dose-response.

NB: The early response genes may take minutes to hours to express the effects of a particular drug, whereas the late response genes may take hours to days for the same. Normal (non-transformed) mammalian cells grow in flat 2D

cell culture containers, forming a monolayer and getting prompt drug access. Contrary to this, in a healthy body, physiological conditions are present within tissues and organs of complex architecture growing as multiple layers. Therefore, growing the cells in 3D cell culture containers and animal experiments is highly essential after the basic 2D cell culture experiments.

4 Mammalian Cell Culture in Cancer Research

Cancer is the uncontrolled proliferation (division by mitosis) of cells, culminating in an uncontrolled cell mass. This infers that cancer cells exhibit uninterrupted cell cycle progression and divide uncontrollably with a negligible or no G0 phase. In the G0 phase, cells do not perform any task about cell division, including preparation for cell division. Perhaps, that's why this phase is sometimes also called as resting phase. The normal non-transformed cells can be transformed into a tumor/cancer cell because of their genetic and/or epigenetic alteration, leading to characteristic genotype and phenotype distinctions. Various immortal cancer cells are now available that can be in vitro cultured to understand distinctive identifying features of cancer cells. A separate chapter (chapter 10) herewith describes the culture of various cancer cells. Briefly, from the cultured cells, the following information may be collected (Masters 2000; Mirabelli et al. 2019).

4.1 Understanding the Phenotype and Genotype of Cancer Cells

The reason for the uncontrolled proliferation of cancer cells is either **genetic** or **epigenetic alteration**. Mutation in the gene sequence and alteration of chromosome number are the major reasons for genetic alteration. Besides spontaneous and induced reasons for mutation, transposons or retrotransposons may generate mutations. Changes in the acetylation and methylation of DNA are the major epigenetic alteration of a cancer cell. Karyotype changes lead to phenotype changes such as irregular in shape, varied in size, large nucleus (may be due to more number of chromosomes), etc. Cultured cells are the major resources to understand all the characteristic alterations of cancer cells both genotypically and phenotypically.

NB: Characteristic alterations or hallmarks of cancer are described in Chapter 11.

4.2 Understanding the Process Through Which a Normal Cell Can be Transformed into a Cancer Cell

In a research laboratory, under an experimental setting, a normal cell can be transformed into a cancer cell via stimulation either with various cancer-causing viruses called **oncoviruses** (e.g., DNA viruses) or oncogenic chemicals (e.g., methylcholanthrene, aflatoxins, asbestos, arsenic, etc.) or radiations (e.g., ultraviolet

radiation). While animal models (particularly syngeneic or immunocompromised and knockout mice models) are the first choices for generating cancers, particularly xenograft mice models presently normal cell culture models are also utilized to generate cancer cells as well as understand the transformation mechanism.

4.3 Understanding the Role of Various Cell Cycle Phases in Regulating Cancer Cell Proliferation

The cell cycle is a normal physiological phenomenon in the life of a mammalian cell. It regulates and decides as and when a cell may undergo division (cell proliferation or growth) and how many times a cell has to divide. A typical cell cycle consists of a repeated cycle of **G0-G1-S-G2-M phases**. In the G0 phase, the newly divided cells undergo complete rest and prepare for the nutritional and physical requirements of cell division. While in the G1 phase, a cell synthesizes all the proteins (enzymes such as polymerases) necessary for DNA replication, in the S phase, DNA replicates (formation of two DNA from one DNA via a semiconservative model). In the G2 phase, cells check their preparation (vis-à-vis DNA, RNA, proteins, and cellular constituents) for cell division to an adequate extent, and finally, in the M phase, the actual cell division or mitosis happens, followed by cytokinesis, thereby completing the cell division.

According to *Hayflick*, all normal cells of a mammalian body (except neuronal cells or mature erythrocytes) undergo finite cell cycles and therefore a limited number of cell divisions. For example, endothelial cells have a capacity of around 50 divisions. Shortening of telomere after every division may be one of the reasons for a limited number of mammalian cell divisions. Following every cell division, each cell enters the G0 phase, the duration of which may vary from one to another cell type. An exception may be neuronal cells (always in the G0 phase) or mammalian mature erythrocytes (do not contain any nucleus), owing to which these cells never enter the cell cycle.

While in a G0 phase, cells receive a sufficient amount of positive signals from molecules such as growth factors, hormones, amino acids, etc., and the expression of cell cycle inhibitory or negative signaling molecules such as **P^{53}**, **P^{21}**, **P^{27}**, **PTEN**, **pRb**, etc. declines to their basal level. The G0 phase cells transit to the G1 phase, which is followed by S and G2 phases, respectively, and finally cells enter the M phase. After the division, the two newly divided cells once again go through the same cycle of Go-G1-S-G2-M, and this cycle will go on and on, till a normal cell is capable of further dividing. However, a cancer cell has no G0 phase or very short G0 phase and exhibits a continuous cell cycle. This means one division ends and immediately prepared for the next cell division begins, i.e., starting cycle for cancer cells.

Cultured mammalian cells are widely utilized to understand the cell cycle, about which some prominent utilities are as ahead:

- Checking the cell proliferation by **MTT** or other cell proliferation assays.
- Understanding cell synchronization in a particular phase of the cell cycle. In synchronization, all cells in a particular population will be in the same cell cycle phase. This means all the cells will be in either G1 or S or G2 or M phase. Synchronization effectively helps to understand the role of various newly synthesized drugs, toxins, etc. on the cell cycle. This is indeed very helpful in new drug discovery.
- Understanding the role of various cyclins and cyclin-dependent kinases (CDKs) on the cell cycle. While cyclins are regulatory subunits of a cell cycle regulatory enzyme, CDKs are catalytic subunits of the same enzyme. In a mammalian cell, there are several cyclins (**cyclin D**, **cyclin E**, **cyclin A**, and **cyclin B**) regulating the cell cycle phases. Similarly, various CDKs such as **CDK4**, **CDK6**, **CDK2**, and **CDK1** catalyze the mammalian cell cycle. Cyclins (regulatory subunit) and CDKs (catalytic subunit) together comprise the complete cell cycle regulating enzymes.
- Experimentally controlling cyclins or CDKs or both may help to regulate the cell cycle. For example, estrogen stimulates cyclin D1 synthesis and therefore activates the G1 phase of the cell cycle. So, attenuation of estrogen-dependent cyclin D1 synthesis may alter the cell cycle pace and arguably, the estrogen-dependent breast cancer as well.

4.4 Understanding the Process of Programmed Cell Death in Normal (Mortal) and Cancer (Immortal) Cells

Programmed cell death or **apoptosis** is a normal natural physiological phenomenon having paramount importance in various processes such as developmental biology and morphogenesis, renewal or replacement of old damaged/aged cells with new cells, etc. Every normal dividing cell loses the capacity for division after a certain number of divisions, and eventually these cells die by the process of programmed death or apoptosis. Apoptosis is regulated by the controlled regulatory activities of proapoptotic proteins such as **Bad**, **Bax**, **Bid**, etc., or antiapoptotic proteins such as **BCl_2**, **Bcl-xL**, etc. While proapoptotic proteins activate apoptosis, antiapoptotic proteins forbid unnecessary apoptosis. The proapoptotic proteins may regulate several procaspases (inactive proteolytic enzymes which act as precursors of active enzymes) which upon activation are converted to **caspases** (active proteolytic enzymes). These enzymes execute a signaling cascade through apoptosis (e.g., inactive procaspase 3 will be converted to active caspase 3). However, cancer cells have a defunct apoptosis mechanism because of mutations in multiple apoptosis-activating proteins such as P^{53}. Cultured mammalian cells are not only used to understand apoptosis but also to observe the effects of various pro- or antiapoptotic molecules. One of the mechanisms through which cancer biologists try to control the cancer cell population is via apoptotic induction in these cells.

4.5 Understanding the Effect of Various Experimental Anticancer Drugs on the Cultured Cancer Cells

Cell cycle proteins such as cyclins and CDKs regulate the proliferation (division) of cells. Various cells in a tissue or an organ are in distinct cell cycle phases and can be separated based on their specific phase using flow cytometer or cell sorter. Each of the cell cycle phases is regulated by specific cyclins and CDKs. For example, in mammals, the G1 to S phase transition (called **G1-S transition**) is mediated by cyclin D and CDK4/CDK6. By regulating these proteins, one can regulate the cell cycle as well as uncontrolled cellular proliferation. In vitro cell culture experiments showed that while the G1 to S transition regulates the cell cycle initiation, the **G2-M transition** checks whether every material necessary for cell division is present to an adequate extent. To check the effects of an experimental drug on the specific phase of the cultured cells, synchronization of cells at a particular cell cycle phase is highly essential (synchronization indicates that experimentally all the cells must be in the same phase of the cell cycle during experimental drug/chemical treatment). It is pertinent to note here that **serum starvation** and **aphidicolin** halt the cell cycle at the G1 phase; **colchicine** and **nocodazole** do so at the M phase, and **5-fluorodeoxyuridine** halts the S phase of the cell cycle. Without the experimentally cultured cells, it is impossible to synchronize mammalian cells under in vivo conditions or inside the body.

5 Mammalian Cell Culture-Driven Vaccine Production

Vaccination is the twentieth century's greatest public health triumph. It is the process of conferring protection to a mammalian body against a disease-causing pathogen or more specifically pathogen-generated molecules either by administering heat-killed or attenuated whole pathogen or a part of a pathogen. Vaccines are either **prophylactic** or **therapeutic**, with the former being used entirely against a physiologically normal host and the vaccine itself also cannot cause any diseased condition in the host's body. Upon administering a prophylactic vaccine, the host organism (immunological cells) produces memory B cells and memory T cells. These memory B and T cells can be stored for years in the secondary lymphoid organs such as the spleen, lymph nodes, mucosa-associated lymphoid organs (**MALT**), gut-associated lymphoid organs (**GALT**), etc. In the event of any subsequent attack by the same pathogen/antigen on the host's body, the memory cells against that particular pathogenic antigen readily recognize the pathogen/antigen and through a series of immunological reactions, effectively eliminate the pathogen/antigen. In therapeutic vaccination, the mammalian subject is already infected with a pathogen, and administering the vaccine to the subject either neutralizes or eliminates the pathogen/antigen. In therapeutic vaccination, since there is no formation of memory cells, the effect of the therapeutic vaccine is transient **or for a short duration only**. It infers that the therapeutic vaccine must be used every time the same pathogen enters the mammalian body and the vaccine is highly useful to neutralize or destroy the

pathogen as long as there is no mutation in the epitope of the pathogen (antigenic drift/antigenic shift) against which the vaccine was produced (Plotkin et al. 1969; Montagnon et al. 1981).

Vaccination eradicated smallpox, lowered the global incidence of polio by 99% since 1988, and achieved dramatic reductions in measles, diphtheria, whooping cough (pertussis), tetanus, and hepatitis B. Approximately **20 vaccines** are currently in use, contributing a small proportion ever since the first human vaccine was developed by **Jenner in 1796** until today. This indicates the substantial major difficulties encountered to develop new vaccines with a large number of trials and clinical assays needed to ensure maximum safety and efficacy for patients. Vaccines for polio, measles, mumps, rubella, chickenpox, and more recently rotavirus and human papillomavirus (HPV) are currently being manufactured using cell cultures. Mammalian cells are used either as a host of the virus to be cultured or as a factory for producing recombinant proteins from the virus.

In general, vaccine generation against the influenza virus is accomplished using a chicken egg-based system and not using mammalian cell culture. Due to the H5N1 pandemic threat, research attempts involving cell culture for influenza vaccines are currently being funded by the US government, including the use of mammalian, avian, and insect cell-based processes as well as vegetable cell line and plants. Presently, a large number of scientists throughout the world had worked very hard to develop vaccines against COVID-19 in a relatively short duration and are continuing to develop the new COVID-19 vaccine, particularly for babies, toddlers, and young teenagers. Of note, the COVID-19 pandemic is caused by a single-stranded RNA virus, called SARS-CoV-2. As a consequence, currently, all paths are open, including novel insights from the recombinant DNA-based vaccines (made with new vectors) and the use of novel adjuvants. However, the removal or inactivation of adventitious viruses remains a unique challenge. Risk assessment related to cell culture techniques is more or less the same as identified in the 1950s.

Among the advantages of using cell culture-based manufacturing processes to produce vaccines include the following:

- The capability for manufacturers to increase vaccine production with ease.
- This enables vaccine production to be enhanced in proportion to easily meet any sudden increase in demand for vaccines such as in the event of a pandemic.
- The ability to produce vaccines faster.
- The production time of vaccines using a cell culture-based process reduces vaccine generation time by half about the embryonic egg process.
- The reduced possibility of viral culture mutation during the manufacturing process.
- Virus mutation can result in culturing vaccine failure and this may be possible while using embryonic eggs.
- Cell-based vaccines are cultured in biosafety level 3 (**BSL3**) conditions.

Table 1 describes the various pathogens and the corresponding cell lines susceptible to them. It is worth noting that there is a single pathogen that could be the infection source for more than one cell line, inferring a likelihood of similar and dissimilar structure-activity relationships (SARs)

Pathogen/virus	Susceptible cell line
Herpes simplex	Vero, HepG-2, human diploid (HEL, HEK), human amnion
VZV **CMV**	Human diploid fibroblasts HepG2 and HEK
Adenovirus	Human diploid fibroblasts HepG2 and HEK
Poliovirus	MK, BGM, LLC-MK2, human diploid, Vero, Hep-2, rhabdomyosarcoma
Coxsackie B	MK, BGM, LLC-MK2, Vero, hep-2
Echo	MK, BGM, LLC-MK2, human diploid, Rd
Influenza A	MK, LLC-MK2, MDCK
Influenza B	MK, LLC-MK2, MDCK
Parainfluenza	MK, LLC-MK2
Mumps	MK, LLC-MK2, HEK, Vero
RSV	Hep-2, Vero
Rhinovirus	Human diploid (HEK, HEL)
Measles	MK, HEK
Rubella	Vero, RK13

MK, monkey kidney cells; **BGM**, buffalo green monkey kidney cells; **LLC-MK2**, rhesus monkey kidney epithelial cells, **HEK**, human embryonic kidney, **RK13**, normal rabbit kidney epithelial cells; **MDCK**, Madin-Darby canine kidney cells, **VZV**, varicella-zoster virus; **CMV**, cytomegalovirus

- This biosafety level provides an isolated environment to ensure safer generation and handling of pathogenic viruses without escaping into the environment.
- People who are allergic to vaccines made from chicken eggs may not suffer similar allergies to vaccines made from cell cultures. Table 1 describes the susceptible cell lines of some widely used pathogenic viral stains, vis-à-vis manifested infection propagating tendencies.

However, the cell-based manufacturing process has the following disadvantages, which limit its application feasibility:

- The relatively higher manufacturing costs. This may translate to more expensive vaccines. This process produces fewer viruses for vaccine manufacturing.
- The volumetric yield of cell-based flu virus is about fourfold lower than the egg-based process. This means a requirement for a larger bioreactor volume along with a much higher capital investment for the production plant. This will further add to the vaccine cost.
- This production method is relatively new in comparison to the process using embryonic eggs. Prominently, there is a lack of long-term safety or rare adverse event data.

5.1 Propagation of Virus in the Cultured Mammalian Cells and Vaccine Production

The propagation and vaccine production against a virus involves the following steps:

Step 1: Cell line selection and culture
Step 2: Propagation of virus in the cultured cells
Step 3: Harvesting and purification of viruses
Step 4: Inactivation and splitting of viruses
Step 5: Formulation of vaccines (adding adjuvants, preservatives, stabilizers)
Step 6: Quality control/quality assurances and batch release

Here is a brief description of the above steps:

5.1.1 Step 1: Cell Line Selection and Culture

- Generally, continuous cell lines like Vero, HepG2, MDCK, CHO, etc. are the best options for viral cultures.
- The cell line is produced in a seed lot system.
- The chosen cell line must be able to propagate the virus in a large quantity and rapid and efficient manner.
- In general virus, DNA should not integrate into the host cell genome (the virus should not activate the lysogenic cycle) (Browne and Al-Rubeai 2007).

5.1.2 Step 2: Propagation of Virus in the Cultured Cells

- Before large-scale virus propagation begins, the virus must be checked for impurities, including other similar viruses and even mutated variations of the same virus. No antigenic drift or antigenic shift should be prevalent.
- The seed must be kept under ideal conditions, usually frozen so that the virus is prevented from becoming either stronger or weaker than desired.
- The virus must be stored in small glass or plastic containers.
- A record of origin, passage history (including purification and characterization procedures), and storage conditions should be maintained for each seed batch.
- Manufacturing begins with the use of small extents of a specific virus as a seed culture. Cell line propagation begins with the small-scale pre-culture propagation of seed cells after thawing.
- The cells are then introduced to the *bioreactor* (bioreactors are used for large-scale cell cultures, inherently for industrial purposes, covered in Chap. 14 of this book) with the selected nutrient medium.
- When the cell line reaches a predetermined cell density, the virus is introduced into the cell line following which it propagates in the cell line.
- Two methods of mass (cell) cultivation are recognized in the industry, namely, microcarrier culture and free cell suspension cultures.
- Both systems begin with cell line cultivation in a bioreactor, which can be further scaled up to thousands of liters.

5.1.3 Step 3: Harvesting and Purification of Viruses

- Product isolation involves the removal of those components whose properties vary markedly from that of desired products.
- Purification selectively separates and retains the desired products at the highest purity as per the pre-determined specification (removing unwanted compounds).
- The most common method of vaccine production is based on an initial bioreactor cell culture followed by purification. Differential centrifugation, filtration, and different modes of chromatography, namely, ion-exchange chromatography, gel filtration chromatography, affinity chromatography, etc., are used to purify viruses.

5.1.4 Step 4: Inactivation and Splitting of Viruses

Viruses can be lipid-coated (enveloped) or non-enveloped. Virus inactivation involves dismantling a virus's ability to infect cells without actually eliminating the virus.

Virus inactivation works by one of the following mechanisms:

- By attacking the virus envelope or capsid and destroying its ability to infect or interact with host cells
- By disrupting the viral DNA/RNA and preventing its replication

The following materials may be utilized for viral inactivation:

A. Solvent detergent inactivation
B. Pasteurization
C. Acidic pH inactivation
D. Ultraviolet inactivation

Here is a brief description of the materials needed for virus inactivation:

A. **Solvent detergent inactivation**
   - Effective with lipid-coated viruses.
   - The detergent used in this method disrupts the interactions between molecules in the lipid coat, rendering the coat dysfunctional and impending replication.
   - Most enveloped viruses cannot live without their lipid coat, so they will die when exposed to these detergents.
   - Other viruses may still live but they are unable to reproduce, making them ineffective.
   - The detergent typically used is Triton X-100.

B. **Pasteurization**
   - This process is effective for both non-lipid and lipid-coated viruses.
   - Because pasteurization involves increasing the temperature (63 °C for 30 min) of a solution to a limit that denatures the viral capsid proteins, it does not matter whether the virus is lipid-coated or not.

C. **Acidic pH inactivation**
- A most effective method for lipid-coated viruses.
- Acidic condition inactivates the virus.
- Incubation typically occurs at a pH of 4 and lasts anywhere between 6 h and 21 days.

D. **Ultraviolet ray inactivation**
- UV rays (wavelength 260 nm) can be used to inactivate viruses since virus particles are small and UV rays can access the genetic material, including the thymine dimerization (TMD) of the same DNA strand.
- Once the DNA dimerizes, the virus particles cannot replicate their genetic materials.
- In TMD, the thymine nucleotides of the same DNA strand join together and prevent the DNA to replicate.

5.1.5 Step 5: Formulation of Vaccines

The most important constituent of a vaccine is the particle/molecule/protein isolated from the microorganism/virus. This specific particle/molecule/protein needs to be purified from the lysed virus using various types of chromatography, electrophoretic separations, etc. The other components comprise suspending fluids. The final formulation must be thoroughly devoid of whole or live viruses.

Preservatives, Stabilizers, Antibiotics, and Antimycotics for Immunogenic Retainment of Vaccines

- **Monosodium glutamate (MSG) and 2-phenoxy ethanol** are used as stabilizers in a few vaccines to help the vaccine remain unaltered on being exposed to **heat**, **light**, **acidity**, **or humidity**.
- **Antibiotics** are added to some vaccines to prevent the growth of microorganisms. The most frequently used antibiotics include neomycin, streptomycin, polymyxin B, chlortetracycline, and **antimycotic** amphotericin B.
- **Thimerosal** may also be used in the containers of some vaccines as a preservative.

Adjuvants or Enhancers

- Aluminum gels or salts (alum).
- Alum is used in several licensed vaccines including *H. influenzae*, inactivated poliovirus, hepatitis A virus, etc.

Inactivating Agents

- Formaldehyde is used in the formulation of some vaccines such as influenza, diphtheria, polio vaccines, etc.

NB: In general, formaldehyde is removed before packaging vaccines owing to its toxicity to human cells.

β-Propiolactone is used against the rabies virus.

Table 2 The various quality control and quality assurance procedures to determine the optimum biochemical state of grown microbial cultures for obtaining an efficient and desired quality product

Test	Purpose of the test
Sterility	Ensures that no microorganisms are present in the product
Chemistry	Ensures that the product has the correct amount of adjuvant and preservative with optimum pH
Safety	Demonstrates that the overdose of the product causes no harm
Residual Toxicity	Validates that the product contains no harmful material
Efficacy	Ensures that each antigen in the product meets the recommended guideline level or better in the internationally recognized test

5.1.6 Step 6: Quality control and Batch Release

Table 2 describes various quality control/quality assurance tests along with their screened parameters.

Interference Test

For products having two or more antigenic components, the test must confirm null interference between individual components, that is, one component suppressing the protective immunological response to another.

Consistency of Production

Before marketing approval of any product, each establishment should produce three consecutive production batches/serials of the completed product (in its facilities) to evaluate the production consistency.

Stability Tests

Stability studies (based on an acceptable potency test) are required to establish **the validity of the expiry date** that appears on the product package.

Batch/Serial Release for Distribution

Before release, the manufacturer must test each batch/serial for the following:

Batch/serial purity test
Batch/serial safety test
Batch/serial potency test

Ahead is a brief discussion about them:

Batch/Serial Purity Test

Purity is determined by testing for a variety of contaminants. Tests to detect contaminants are performed on master seeds, primary cells, master cell stock, ingredients of animal origin if not subjected to sterilization (e.g., fetal bovine serum (FBS), bovine albumin, etc.), and each batch of the final product before release.

Batch/Serial Safety Test
Batches are considered satisfactory if local and systematic reactions to vaccination with the batch to be released are in accord with those described in the registration dossier and product literature.

Batch/Serial Potency Test
A batch/serial potency test is required for each batch before release. These tests are designed to correlate with the host animal vaccination-challenge efficacy studies.

Other Tests
Depending on the form of vaccine being produced, certain tests may be indicated.

These tests may concern:

- The level of moisture contained in desiccated products
- The proportion of residual inactivated virus in killed products
- Complete inactivation of killed products
- pH
- The extent of preservatives and permitted antibiotics
- Physical stability of the adjuvant
- Retention of vacuum in a desiccated product
- A general physical examination of the final vaccine

Sampling
Sample should be selected from each batch/serial of product. The selector should pick arandom representative sample which could ensure uniformity of standard quality.

Labeling
Standards for labeling products vary from country to country basis (Walter et al. 1992; Werz et al. 1997). Figure 1 summarizes the chronological steps in vaccine production on an industrial scale.

6 Recombinant Protein Production Using Mammalian Cell Culture

Recombinant DNA technology (**RDT**) or genetic engineering is a process through which a specific gene of interest (called an **insert**) is inserted (technically called **ligated**) into a DNA vector for its multiplication (technically called **cloning**) and **expression** (synthesis of desired protein).

In RDT, vectors are specific DNA having a capacity to accept a foreign DNA (which is our gene of interest) for cloning (making multiple identical copies) and expression (synthesis of protein) in a suitable host cell. The protein produced by recombinant DNA technology is called recombinant or chimeric protein.

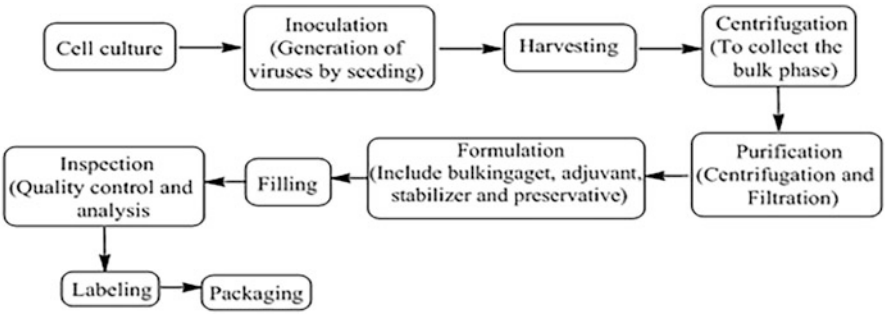

Fig. 1 A schematic description of the vaccine production process. Despite the differential placement of each phase, there is unique importance of every operation in the efficient functioning and timid vaccine availability

A DNA vector is incapable of cloning and expressing the gene of interest, without a host cell. While several different host cells including prokaryotic *Escherichia coli* and single-cell eukaryotic cells such as yeast (e.g., *Saccharomyces cerevisiae*) can be effectively and easily utilized for cloning and expression of various genes, multiple mammalian cells including the Chinese hamster ovary (CHO), human embryonic kidney (HEK), etc. are the best choice as the host for cloning and mammalian gene expression (Wurm 2004; Aricescu et al. 2006; Lai et al. 2013; Hunter et al. 2019).

This section is divided into two parts:

- **F.I.** Process to Produce a Mammalian Protein Using Recombinant DNA Technology
- **F.2.** Major Recombinant Proteins Produced by Mammalian Cell Culture

Here is a brief discussion about them:

6.1 Process to Produce a Mammalian Protein by Recombinant DNA Technology

In this section, the various steps involved in recombinant DNA technology are discussed.

6.1.1 Steps to Produce Recombinant Proteins

Initially, the gene of interest (**GOI or gene of interest: the gene programmed for cloning and recombinant protein production**) that produces the desired recombinant protein must be optimized. The isolation of a specific gene encoding a specific product (**protein**) can be made either from the mammalian genome (**genomic library/cDNA library**) or via specific sequence-driven synthesis. The gene must have the necessary transcription elements for the production of a stable and mature mRNA equivalent to the capacity of producing a complete desired protein (Fig. 2).

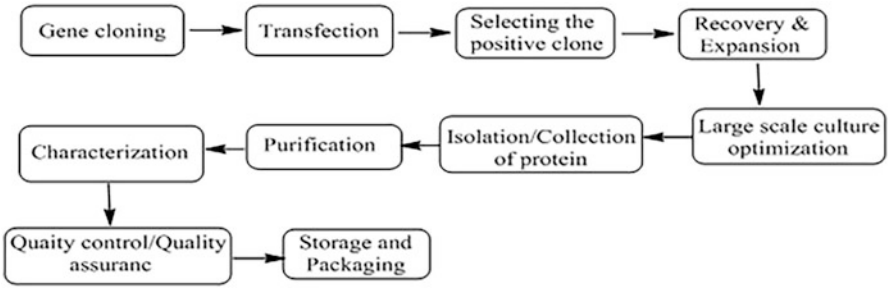

Fig. 2 A schematic representation of a recombinant protein expressed by a mammalian expression system. Post-pilot-scale optimization, isolation, purification, and characterization of target proteins are key steps

While the majority of (~90%) prokaryotes possess only one large DNA constituted of several genes, a eukaryotic cell contains many DNAs. For example, a human somatic cell contains 46 DNAs. In recombinant DNA technology, **gene** is defined as the small unit of DNA having the capacity to produce a single protein (in monocistronic eukaryotes) or more proteins (in polycistronic prokaryotes). **Cloning** implies making multiple copies of the **gene of interest (GOI)/DNA of interest** (technically also called an **insert**) by joining it with an **auto-replicating (self-replicating)** vector DNA to form a **recombinant or chimeric vector**. Thus, adequate standardization and characterization of a suitable vector are necessary for screening the ability to incorporate a foreign DNA, making it multiple copy clones and express the GOI in the in vitro cultured mammalian cells. In addition, a second gene present either in the same or different vector is transferred, conferring a selective advantage to recipient cells before transferring the recombinant vector into a suitable host cell (Schimke 1984; Omasa 2002).

The **GOI expression** is host-specific since a vector (a single carrier) or a recombinant vector (intentionally driven) needs a host cell for its multiplication and subsequent expression. Today, there is a wide range of expression systems available for large-scale recombinant protein production. These expression systems include *Escherichia coli*, baculovirus-mediated insect cell expression, yeast, and several mammalian-based systems. Each vector has its respective advantages concerning cost, usage simplicity, and implicit posttranslational modification profiles. In the presence of the selection agent, (generally administered after a few days of gene transfer), only those cells that express the selector gene survive.

Transfection involves the transfer of naked recombinant vectors/DNA to compatible mammalian host cells. In today's RDT experiments, various transfection procedures are used such as **transformation** (involving *Escherichia coli* as a host cell), **electroporation** (use yeast and hard to transfect host cell), or just simple **lipid-based transfection reagents** (e.g., **lipofectamine**) or other transfection reagents (e.g., **transfectamine**) or **nanoparticle-based transfection reagents** (**Sigma's N-TER™**) are used for mammalian cells.

After transfection of the host cell line with the expression vector containing the **GOI** and selection marker, the host cells grow and undergo drug selection enabling the screening of the desired host containing the recombinant vector. When gene amplification systems are used, concentrations of selection agents (e.g., **MTX** or **MSX**) can be increased stepwise to derive more productive cell clones. Cell clones with high recombinant protein titer are chosen for progressive expansions before cell banking and further clone evaluations such as production stability of the cell clones and quality of recombinant protein.

Following selection, surviving cells are transferred as single agents to a second cultivation vessel, and the cultures are expanded to produce clonal populations. Eventually, individual clones are evaluated for recombinant protein expression, with the highest producers being retained for further cultivation and analysis. From these candidates, one cell line with the appropriate growth and productivity is chosen for generating the recombinant protein. A cultivation process is then established as per the production requirements. So far, all mammalian recombinant therapeutics are naturally secreted proteins or have been developed from gene constructs involving protein secretion using rough surface endoplasmic reticulum (ER) and Golgi apparatus secretory pathway.

Here is the explanation of the steps involved in cloning and expression of various recombinant proteins using mammalian host cells:

1. Optimization of gene of interest
2. Selection of vector and optimization of mammalian expression system
3. Selection of host cells
4. DNA delivery and integration: transient and stable transfection
5. Selection and optimization of positive clones
6. Selection and optimization of cell culture medium
7. Selection and optimization of cultivation mode: adherent vs nonadherent (perfusion and fed-batch)
8. Purification of the expressed protein
9. Quality control and quality assurances

Here is the step-by-step discussion of the above points:

Optimization of Gene of Interest

Gene is defined as a structural and functional unit of DNA. DNA consists of a linear sequence of many nonoverlapping genes. In humans, while the somatic cell contains 46 DNA, the reproductive or germ cell (sperm/ovum) contains only 23 DNAs. Every DNA has a specific number and a characteristic length and a proportionate varying number of distinct genes. Structurally, a mammalian gene includes the **coding sequence** (exons) for a protein the **noncoding sequence** (introns), having a regulatory role, the **promoter** (the region binding with the RNA polymerase to initiate transcription), and the **terminator** (the region which ends transcription). Functionally, a gene transfers genetic information from one generation to the other in the form

of mRNA (synthesized via **transcription**), followed yet again by the synthesis of protein through **translation**.

To clone a small GOI, one can synthesize it artificially. However, to get a large GOI, one may use either a cDNA library or a genomic library. Due to the enormous size of the mammalian genome, the larger genomic library (**a collection of genes in suitable vector and collective representation of the entire genome**) is used to clone mammalian genes. Indeed, it is a mammoth task to identify any GOI from the genomic library. Therefore, isolation of the GOI from the cDNA library (collection of protein-coding exons only in a suitable vector, representing all mRNAs) is the best choice.

For preparing a cDNA library, mRNA is purified from cells using **oligo-(dT)** cellulose chromatography. The mRNA molecules bind to the oligo-(dT) linked to the cellulose column via polyA tails, while the remainder of RNA species flow through the column. The bound mRNAs are then eluted from the column. When the mRNA has been purified, double-stranded DNA must be synthesized from the cDNA.

One can choose any of the following sources to collect mRNA:

- Gonadotrophic cells of the anterior pituitary produce follicle-stimulating hormone (**FSH**).
- Human kidney cell line HT1080 secretes urokinase.
- Endothelial cells from blood vessels synthesize tissue plasminogen activator (**tPA**).
- Liver hepatocytes synthesize blood clotting factors like **clotting factor VIII**.
- Pancreatic islets synthesize hormones like **insulin**, **glucagon**, and **somatostatin**.
- Duodenal cells synthesize epidermal growth factor (**EGF**).

While a GOI can be collected and isolated from the somatic cells present in any of the mammalian organs (because technically all the somatic cells contain the same gene or DNA), a specific cell type may be preferred because of the exact **ORF** (an ORF is a continuous stretch of codons beginning with a start codon (usually AUG) and ends at a stop codon (usually UAA, UAG, or UGA) of the GOI in specific somatic cells). In its native context, a GOI in other tissues may contain secondary structures, mRNA that might inhibit ribosome processing, alternative splicing sites, sequence elements signaling mRNA degradation, or codons that are rarely used in an expression host. Therefore, the variables that affect the ORF of the GOI and its ability to produce large extents of proteins in mammalian cells can be numerous. De novo DNA synthesis provides the researchers with the ability to create a full-length gene considering these parameters and controlling the presence or absence of specific restriction sites or motifs.

In addition to the optimization of the gene associated with the recombinant protein of interest, one must consider the subcellular location of the protein. The widespread use of mammalian cells has been dominated by the production of secreted proteins that require a signal sequence directing the synthesis of needed protein outside the cell.

The secretory proteins are produced by the rough surface endoplasmic reticulum **(RER)**. While the protein synthesis is initiated on the ribosomal surface and continues, the amino terminus (leader sequence) of the de novo generated protein binds with the transporter of the signaling sequence (**TRAP**) and enters into the lumen of RER through **sec 61** (a pore-forming protein on the RER surface). In the RER, complete folding (secondary to quaternary structure) and posttranslational modifications (including advanced N-linked glycosylation) of the protein take place before being secreted toward the cis-Golgi apparatus. Typically, RER secreted protein has a signaling sequence that binds with various vehicles (named as COP1, COP2, etc.) and enters the Golgi apparatus. In the Golgi apparatus, the protein is further modified (e.g., O-linked glycosylation), undergoes maturation, and is finally secreted from the trans-Golgi apparatus for the specific destination (may be any subcellular compartments including the membranes or extracellular space). For secretory proteins, the protein must be secreted into the extracellular space. Of note, the signaling sequence present in the protein determines its implicit final destination.

It has been reported that the expression of a recombinant protein does not always correlate with its mRNA levels, and a rate-limiting step can be the secretory pathway. Intuitively, the best choice of a signal sequence may be the protein's native signal. However, testing a panel of commonly used signal sequences is desirable. Not surprisingly, the signal sequence can have a dramatic effect on protein productivity, with even fourfold enhanced expression levels.

Recombinant proteins of mammalian origin are commonly expressed in eukaryotic expression systems, particularly human cells as the host of choice to ensure the formation of **disulfide bridges** and **proper glycosylation**. Antibody and antibody-like molecules are examples of proteins that are relatively easily made in mammalian cells and are easily purified by protein A or **protein G** resin-dependent affinity chromatography. However, some recombinant proteins, sub-domains, and mutant protein versions can be plagued with issues regarding **misfolding**, **aggregation**, or entirely lacking expression. **Fusion tags** are frequently used to address such issues.

Selection of Vector and Optimization of Mammalian Expression System

A **vector** is a DNA molecule having the ability to accept any foreign DNA and spontaneously replicate it as and when it will replicate its DNA inside the host cells. As described in previous paragraphs, in molecular cloning, the foreign DNA has termed a gene of interest or **GOI**. So, a vector can make multiple copies of the GOI inside a suitable host cell. For expressing heterologous genes in mammalian cells, usually, vectors derived from mammalian viruses are used (Sambrook et al. 1989; Yarranton 1990; Kost and Condreay 2002).

A mammalian expression vector must have the following sequences:

- Characteristics of normal cloning vectors such as the origin of replication, multiple cloning sites for insertion of GOI, unique restriction sites, selectable maker(s), and auto-replicating capacity.
- Contains a eukaryotic origin of replication from an animal virus, e.g., Simian virus 40 (**SV40**).

- May contain sequences for propagation in the prokaryotic host (*Escherichia coli*) and origin of replication from *Escherichia coli* (usually acts as a shuttle vector).
- The GOI must contain a start codon.
- A vector must contain an efficient promoter sequence for high-level transcriptional initiation. A **strong promoter** is essential to drive the expression of product gene(s). A strong promoter indicates a region of DNA has a better and stronger affinity with the RNA polymerase. Viral genetic elements are used to construct several eukaryotic expression vectors. Viruses are highly efficient replicators and viral gene expression is adapted to eukaryotic systems. Therefore, the strategy has been to use the regulatory elements of the viral genome, i.e., promoters, enhancers, polyadenylation signals, introns, replication origins, **IRES elements**. Moreover, the use of transcription control regions is also important. Very strong promoters, small introns (e.g., CMV intron), and regulatory elements are often constitutive and require only host transcription factor binding. Promoters such as those derived from Simian virus 40 (SV40) early promoters, the Rous sarcoma virus (**RSV**) long terminal repeat promoters, and cytomegalovirus (**CMV**) immediate early promoters constitutively drive the gene expression placed under their control. Inducible systems can also be used such as heat shock protein (**HSP)** inducible promoters, glucocorticoid hormone inducible promoters, and several others. However, the promoter sequences in general suffer leaky gene expression. Of note, nonviral promoter, such as the elongation factor (EF)-1, is also known.
- The vector must contain a transcription start site.
- The vector must contain a ribosome binding site (**Kozak sequence**, for eukaryotic/mammalian cells).
- The use of transcription control regions in the vector is also vital (Fig. 3).
- A vector must contain **mRNA processing signals**. Codon optimization for the target cell type, GC/AT ratio balancing, and signal sequence optimization have been demonstrated to accelerate mRNA processing and improve secretion.
- Transcription termination sequences: **Adenylation signals** from animal viruses, e.g., SV40, are essential to add to the vector.

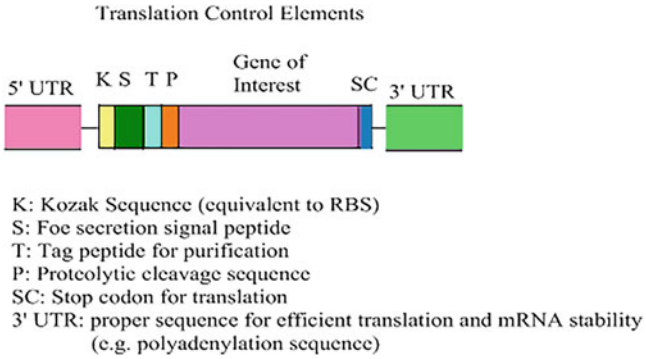

Fig. 3 Pictorial depiction of mammalian translation control elements in an eukaryotic vector

- The vector must contain certain selectable markers and promoter sequences that drive the selectable marker gene(s). The common approach used in generating cell lines for producing therapeutic proteins relies on gene amplification induced by a selective marker such as dihydrofolate reductase (DHFR) or glutamine synthetase (GS). Bacterial gene neo (encoding neomycin phosphotransferase) confers resistance to G418 (geneticin, G418 sulfate). When DHFR is in use, the recipient cell must have a defective DHFR gene which makes them unable to grow in the presence of methotrexate (MTX), unlike transfected cells having a functional DHFR gene.
- Besides gene-targeting technology, chromatin opening elements and attachment regions should also be incorporated into vector genetic machinery to augment the final product production.
- Matrix-attachment regions.
- Chromatin insulators and locus control regions.
- Vectors must also possess the elements that create a genomic environment for high transcriptional activity (positional independence).
- Targeting of expression vector to the transcriptionally active site in the genome is accomplished using homologous recombination.
- Examples of mammalian expression vectors include the adenoviral vectors, the **pSV** (a plasmid vector designed for the expression of cloned sequences in mammalian cells driven by the **SV40** early promoter and enhancer), and the **pCMV** (a plasmid vector containing **cytomegalovirus** immediate early promoter) series of plasmid vectors, **vaccinia** and **retroviral** vectors (permanently

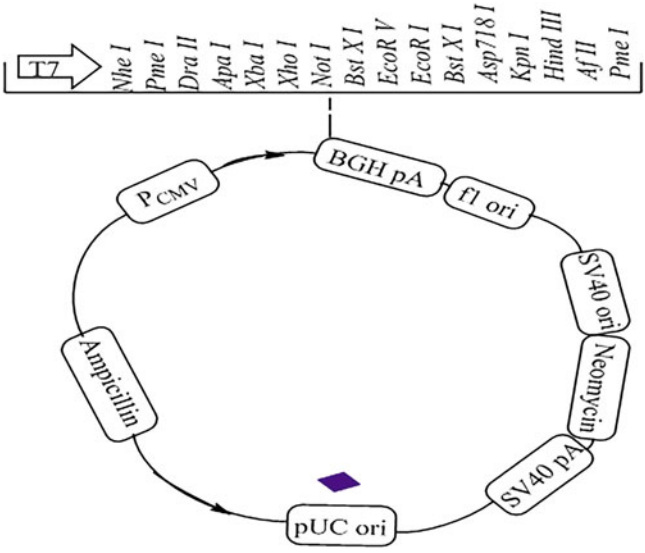

Fig. 4 Characteristic molecular features of a mammalian expression vector. The vector contains CMV promoter, multiple cloning sites with unique restriction sites, SV40 promoter, neomycin, ampicillin-resistant genes, PUC/PBR322 bacterial plasmid origin of replication, etc

integrate the GOI into the host cell genome), and **Baculovirus** (DNA viruses that infect insect cells) **vectors** (Fig. 4).

Usefulness of Mammalian Expression Vector

Mammalian expression vectors offer considerable advantages (for the expression of mammalian proteins) over bacterial expression systems. The advantages of the mammalian expression system are as follows:

Mammalian expression vectors exhibit **proper folding**, **posttranslational modifications**, and **relevant enzymatic activity.** These may also be more desirable than other eukaryotic nonmammalian systems (e.g., yeast vectors such as **YAC**) whereby the expressed proteins may not contain the **correct extent of glycosylation**. These are of particular significance in producing membrane-associating proteins that require chaperones for proper folding and stability besides being comprised of numerous posttranslational modifications. The downside, however, is the **low yield of product** compared to prokaryotic vectors as well as the costly nature of the optimization and characterization techniques. The complicated technology and potential contamination with animal viruses of mammalian cell expression also manifested a constraint on their use in large-scale industrial production.

Drawbacks of Using Mammalian Expression Vector

The drawbacks of using mammalian expression vectors are as below:

- Complicated technology involves complex requirements.
- Possible contamination of animal viruses.
- Generally, high cost is involved in mediating recombinant protein expression within the mammalian expression system.

6.1.2 Selection of Host Cells

For recombinant mammalian protein generation, mammalian cells are the first choice for recombinant vector transfection and subsequent recombinant protein expression. Mammalian host cells have several advantages for mammalian protein production when compared with single-cell eukaryotic yeast cells or even prokaryotic cells such as *Escherichia coli* (Browne and Al-Rubeai 2007).

The potential advantages of using mammalian cells as host cells are as follows:

- Efficient intron removal using splicing (if used gene is from genomic library instead of cDNA library)
- Mammalian/human-specific posttranslational modifications
- Highest functionality due to posttranslational modification
- High compatibility concerning humans
- Low immunogenicity to humans
- Safe to use

However, the use of mammalian cells includes some drawbacks also. The drawbacks of using mammalian cells as hosts are as follows:

- Mammalian cells are fragile and sensitive to shear stress.
- Slow growth.
- Mandate fastidious growth requirement.
- Selection take up time.
- Expensive culture techniques.
- Difficult to achieve transfection of gene of interest (low transfection efficiency).
- Risk of animal viruses contamination.
- Stringent control is required for screening contaminated viruses.
- Sometimes specialized cell lines are needed for specific modifications.

Here is a brief description of frequently used mammalian cell lines for producing recombinant proteins.

Mammalian Cell Lines Used in Recombinant DNA Technology

Selected mammalian cell lines widely utilized for recombinant proteins production are as below:

- Chinese hamster ovary (CHO) cells
- NSO cell line
- Human embryonic kidney (HEK) 293 cells
- HT-1080 cell line
- PER.C6 cells

Other mammalian cells used for recombinant protein production are the baby hamster ovary (BHO) cell line, COS cell line, HeLa, A549, MCF-7, HepG2, NIH3T3, U2OS, CAD, P19, L929, N2a, Y79, SO-Rb50, DUKX-X11, and J558L.

Ahead is a brief discussion of some of the widely used mammalian cell lines for recombinant DNA technology:

Chinese Hamster Ovary Cells

Regarding the various mammalian cells, Chinese hamster ovary (CHO) cells are the first line of choice for the stable expression of mammalian genes. The approval of CHO-derived tissue plasminogen activator (**tPA**, **activase**) in 1986 revolutionized medicine, raising the possibility of using mammalian cell culture for manufacturing therapeutic proteins. More than 30 years after tPA approval, CHO cells are the predominant hosts used to generate several therapeutic proteins. About 70% of all recombinant proteins produced today are made in CHO cells, including DUXB11, DG44, and CHOK1 lineages. While DUXB11 and DG44 cells do not have dihydrofolate reductase (DHFR) activity, the CHOK1 cells do exhibit endogenous DHFR activity. CHO cells remained the most preferred mammalian cell line for generating recombinant therapeutic proteins for several reasons which are discussed in the following points:

- **First**, while CHO cells are naturally adherent in nature, they are capable of adapting and growing in suspension culture, ideal for large-scale (industrial) culture. In suspension culture, cells grow in larger densities than in the adherent culture.
- **Second**, CHO cells pose less risk as few human viruses can propagate in them.
- **Third**, CHO cells can grow in serum-free (less expensive) and chemically defined cell culture medium, ensuring reproducibility between different cell culture batches.
- **Fourth**, CHO cells allow posttranslational modifications (PTMs) to recombinant proteins which are compatible and bioactive in humans. Specifically, glycosylation of glycoproteins produced by CHO cells is more humanlike, devoid of the immunogenic α-galactose epitope. However, CHO cells are unable to mediate human glycosylation (**CHO cells lack α-sialyltransferase and α-fucosyltransferases**) and produce glycans that are not expressed in humans, namely, **α-gal and N-glycolylneuraminic acid** (NGNA; a sialic acid which is not synthesized by humans). CHO cells can produce **galactose-α-1,3 antigens on proteins**. Circulating antibodies against both of these *N*-glycans are present in humans and are likely to manifest increased immunogenicity and altered pharmacokinetics upon being used in humans.

NB: It was later shown that CHO cells express low NGNA levels and that humans incorporate NGNA into proteins from dietary sources, which tempered immunogenicity concerns.

- However, the attachment of nonhuman glycans may not be a concern for therapeutic proteins which do not require glycosylation, illustrating the importance of considering the specific product on choosing an appropriate cell line for protein production.
- **Fifth**, several gene amplification systems are well established to make use of CHO cell's genome instability and allow the consequent gene amplification, ultimately resulting in higher recombinant protein yield. Currently, recombinant protein titers from CHO cell culture have reached the gram per liter range, a nearly 100-fold improvement over a similar process in the 1980s. The significant titer improvement is substantially due to the progress in the establishment of stable and high-producing clones as well as significant culture process optimization. Due to these reasons, CHO cells are established host cell lines for regulatory approvals of therapeutic glycoprotein products.

NSO Cell Line

The NSO cell line originates from non-secreting mouse **plasmacytoma (myeloma)** cells that have undergone multiple cloning and selection rounds to yield immortalized non-IgG-secreting B cells. However, these cells possess the appropriate machinery for producing and secreting various monoclonal antibodies such as **palivizumab** and **ofatumumab**. Thus, NSO cells are non-immunoglobulin-secreting myeloma cells. These cells can be cultivated in serum and non-serum-containing cultivation

medium and are reasonably amenable to scale up in large cultivation vessels (up to 20,000 L capacity).

However, NSO cells present some drawbacks that complicate their cultivation. Unlike most mammalian cell lines, NSO cells seldom grow in the absence of exogenous cholesterol (behave as cholesterol auxotrophs) and are therefore routinely cultivated in the presence of cholesterol, usually delivered from the serum. Since the presence of animal origin ingredients, as well as other proteins, is undesired in the cultivation medium, it complicates the use of NSO cells due to the difficulty of supplying cholesterol in a protein-free medium as it requires carriers such as **cyclodextrins** to enhance cholesterol "solubility." The development of cholesterol-independent NSO cells has been demonstrated, although with a certain unpredictability that has limited their use.

Recent advances into the mechanism of cholesterol requirement have linked epigenetic gene silencing caused by methylation upstream of the 17-hydroxysteroid dehydrogenase type 7 coding region that catalyzes the **lanosterol** to **lathosterol** conversion, which has been listed as the cause of this deficiency. Although industrial groups have developed cholesterol-independent NSO lineage, no commercial production of mAbs using NSO cholesterol-independent cells is presently being pursued. It is quite possible (given their usage simplicity) that these biochemically and genetically understood cholesterol-independent cell lines could eventually be used for commercial production.

NSO cells lack endogenous glutamine synthetase (**GS**) activity, making them suitable for use with GS as a **selectable marker** for recombinant antibody expression. High antibody productivity has been reported from non-GS NSO cell lines as well. Mouse-derived cell lines, including NSO, produce **N-glycolylneuraminic acid** (NGNA; a sialic acid that cannot be synthesized by humans) at appreciable extents. This sialic acid form was initially believed as a potential immunogenicity concern in humans. Murine cells, including NSO, do produce **alpha-Gal-alpha (1,3) Gal linkages**, which antibodies have been shown to express in humans. Although NSO cells have been used in industry to produce therapeutic antibodies, their potential immunogenicity aspects have likely limited their use for therapeutic antibody production.

Human Embryonic Kidney 293 Cells

In 1973, *Dr. Alex Van der EB's* laboratory from the University of Leiden, Holland, generated a cell line from human embryonic kidney (HEK) cells of an aborted normal human fetus via transformation with sheared adenovirus 5 DNA. In this transformation, a 4.5-kilobase (kb) DNA from the viral genome was incorporated into the human chromosome 19 of the HEK cells. Thus, the HEK name originated from human embryonic kidney cells, and the number 293 was probably the experiment number in Alex Van der EB's laboratory.

HEK-293-T is a human cell line derived from the HEK-293 cell line that expresses a mutant version of the SV40 large T antigen. The HEK293 cell line stably expresses the Epstein-Barr virus nuclear antigen-1 (HEK293-EBNA1, or 293E). These cells are loosely adherent and can also grow as suspension cultures.

This is the most commonly used cell line for large-scale culture owing to its capability of human-specific posttranslational modifications (PTMs). In the past few years, five therapeutic agents produced in HEK293 cells have been approved by the Federal Drug Administration (FDA, USA) or the European Medicines Agency (EMA) for therapeutic use. These agents are drotrecogin alfa (Xigris; Eli Lilly Corporation, Indianapolis, IN), recombinant factor IX Fc fusion protein (rFIXFc; Biogen, Cambridge, MA), recombinant factor VIII Fc fusion protein (rFVIIIFc; Biogen, Cambridge, MA), human cell line recombinant factor VIII (human-cl rhFVIII; NUWIQ; Octapharma, Lachen, Switzerland), and dulaglutide (Trulicity; Eli Lily, Indianapolis, IN).

HT-1080 Cell Line

HT-1080 is a human fibrosarcoma cell line. This cell line was created from tissues taken in a biopsy of a fibrosarcoma present in a 35-year-old human Caucasian male. The patient, who served as a sample source, had not undergone radio- or chemotherapy, making the introduction of unwanted mutations into the cell line a remote possibility. The cell line carries an **isocitrate dehydrogenase 1 (IDH1) mutation** and an activated **N-RAS (RAS: rat sarcoma) oncogene**. This cell line is capable of human-specific posttranslational modifications (**PTMs**) and is used for the production of **agalsidase alpha** (used in Fabry disease), **idursulfase** (Hunter syndrome), **velaglucerase alfa** (type 1 Gaucher disease), etc.

PER.C6 Cells

Among the emerging mammalian cell lines, the PER.C6 cell line appears to be the most advanced in its usage and acceptance. Cells of this cell line are derived from human embryonic retina cells immortalized by transfecting the E1 genes from adenovirus 5 DNA. Like NSO and CHO cells, PER.C6 cells can proliferate indefinitely in suspension under serum-free conditions. PER.C6 cells offer the potential for a humanlike glycosylation pattern with an added advantage of null undesired murine glycans. Several recent communications have disclosed that Per.C6 cells can be cultivated to very high densities at large-scale fed-batch culture, and their capability of supporting enhanced recombinant protein yields up to 8–10 $g \bullet L^{-1}$ of a tested monoclonal antibody (mAb). These promising data are vital aids for the Per.C6 cell line to gain appeal in the next few years as a potential platform for the production of recombinant mAbs or other proteins. Several Per.C6-based products are currently undergoing clinical trials. However, no mAb production platform based on Per.C6 cell cultivation has yet reached the regulatory registration stage. Regulatory concerns exist regarding the use of human-based cell lines for mAb production due to their lack of resistance against adventitious agents.

Additional cell lines such as baby hamster kidney (BHK-21) cells which are used for the production of **factor VIII antigen** are being highly researched. Of note, factor VIII antigen is involved in blood coagulation. African green monkey kidney (or COS) cells are also widely used for transient transfection of mammalian genes.

COS is an acronym, derived from the cells being CV-1 in origin alongside carrying the SV40 genetic material. Another important cell line of use for recombinant protein production is SP2/O. All these cell lines have their advantages and disadvantages and so users must carefully choose them even before starting their research on recombinant protein production.

Process-Related Safety Concerns Using Mammalian Cell Line

For proteins produced in nonhuman as well as in human cell lines, potential safety concerns arise from the possibility of process-related contaminants and immunogenicity.

Process-related contaminants may include infectious agents (viral, bacterial, fungal, mycoplasma, etc.) with the potential to result in host infection, nucleic acid contaminants with the potential to integrate into the host genome (theoretical), and other contaminants from the manufacturing process such as exogenous nonhuman epitopes (e.g., from animal serum used during the manufacturing process) that can be incorporated into human cells and the resultant bio-therapeutic proteins.

However, current manufacturing technologies, typically including multiple viral inactivation or clearance steps (such as nanofiltration), have largely mitigated this concern and supposedly provide more effective viral clearance than observed in CHO cells. Additionally, the recombinant protein must not immunologically react with the host cell or must not interact with the host cells if used therapeutically.

6.1.3 DNA Delivery and Integration: Transient and Stable Transfection

The Methods of DNA Delivery into the Mammalian Cells

In 1973, *Graham and van der EB* showed that exposing cells to nanoparticles of DNA and calcium phosphate facilitates the DNA transfer into cultivated mammalian cells. Now, viral gene transfer remains the preferred approach to generating stable cell lines for manufacturing purposes. Calcium phosphate transfection, electroporation, lipofection, and biolistic and polymer-mediated gene transfer are routinely used and reasonably efficient and reliable transfection methods (Colosimo et al. 2000; Geisse and Henke 2005).

Calcium Phosphate Method

Very briefly the process is as follows: Culture cells in mammalian cell culture grade polystyrene container. Grow the cells within 50–60% confluence. Mix the cells thoroughly with DNA in a phosphate buffer. Thereafter, add the solution of calcium salt, which forms a precipitate. Incubate the calcium-treated mammalian cell culture plate treated with calcium at 37 °C. Cells take up the calcium phosphate crystals including finite proportions of DNA.

Liposome-Mediated DNA Delivery

A liposome could be described as a minute spherical sac of phospholipid molecules enclosing a water droplet, especially as one formed artificially, to carry drugs or other substances inside the tissues. Culture cells up to 50–60% confluence. Mix the DNA

with lipid to form liposomes, small vesicles having some DNA inside. To gain entry into the cells, the DNA-carrying liposomes fuse with the cell membrane and carry DNA within the cell.

Electroporation

Electroporation is a physical transfection method requiring an electrical pulse to create temporary pores in cell membranes through which substances like nucleic acids can pass into cells. Cells grow till 50–60% confluence and then using the electroporator (the machine involved in electroporation) are subjected to electrical shock according to the protocol. It is a highly efficient strategy for the introduction of foreign nucleic acids into many cells, particularly hard to transfect cells because of the thick outer walls such as mycobacteria (*M. tuberculosis*).

Biolistic and Polymer-Mediated Gene Transfer

Gene gun design was **invented** by *John C Sanford*, *Ed Wolf*, and *Nelson Allen* at Cornell University and by *Ted Klein* of DuPont, between 1983 and 1986. The original target was onions (chosen for their large cell size), being used to deliver marker gene-coated particles. The method is also called holistic gene transfer, although its first use was for plant cell transformation. However, the technology was later successfully applied in mammalian cells in vitro as well as in vivo. The incumbent gene transfer is accomplished by bombarding target cells with DNA-coated gold particles driven by a pressurized inert gas such as helium or via high-voltage electronic discharge. Efficient gene transfer necessitates fine optimization of the procedure to maintain penetration capacity, alongside minimizing the tissue/cell damage. Among the parameters that impact the gene transfer efficiency include the microsphere's size and density, bombardment force, gene gun instrumentation, and microspheres to DNA stoichiometry. Typically, these parameters vary with different cells and tissues in animals.

Biolistic gene transfer is advantageous in being fast, simple, and highly efficient. Moreover, the technique is highly suited to deliver a wide range of macromolecules, such as nucleic acids and proteins. It is impossible to assess whether any of the above methods is superior to the others because of the cell-specific suitability and inadequate comprehensive studies.

Mode of Transfection: Transient Versus Stable Transfection

The process of transferring naked GOI or recombinant vector into the eukaryotic host cells is called **transfection**.

Transfection may be of two types:

Transient transfection
Stable transfection

Ahead is a brief description of each regime:

Transient Transfection

- Transfer of **naked DNA** (DNA without any cell)/plasmid DNA into the cytoplasm not integrated into the mammalian host cell genome using any of the above transfection procedures.
- With time the DNA/plasmids may be lost from the progeny because the host cell may not able to replicate the transfected DNA.
- So, the production of recombinant protein may be for small durations (temporary) only.
- Applicability to a wide range of host cell lines, including various mammalian cells.
- Intrinsic genetic stability and consistency due to the extremely short time frame between vector generation and product recovery.
- Suitability to multiple processing, allowing the study of multiple genes or mutants at the same time.
- Simplicity, in particular for the construction of expression vectors (Liu et al. 2008).

Stable Transfection

A selectable marker is included in the plasmid vector so that after the cells have been transformed, the rare cell incorporating plasmid DNA into its genome (through DNA repair and recombination enzymes) can be isolated and cloned (because the progeny of the transformed cell will inherit the plasmid DNA including the selectable marker gene, e.g., antibiotic resistance gene).

Thus, the major criteria of stable transfection necessitate a stable integration of GOI into the host genome, becoming part and parcel of the host genome, and thereafter transferring the coded genetic information from generation to generation (as and when the host genome will transfer its genetic information to the next generation). In this case, the site-specific recombination is such that it will not interrupt or jeopardize the GOI expression as well as the drug resistance/other selectable markers.

Position Effects of Integrated Gene of Interest into the Host Genome

The site of integration of the GOI has a major effect on the transcription rate of the recombinant gene (a phenomenon known as the position effect). Integration into inactive heterochromatin results in little or no transgene expression, whereas integration into **active** euchromatin readily allows transgene expression.

However, integration into euchromatin may not be adequate to ensure the long-term expression of the recombinant gene. Transgene expression in mammalian cells is rapidly inactivated (silenced) in many cases, substantially due to the influence of neighboring condensed chromatin. Gene silencing correlates with histone hypoacetylation, lysine of histone H3 methylation, and an increase in CpG methylation within the transgene promoter region.

Several strategies have been demonstrated to overcome the negative position effects of random integration. Protective cis-regulatory elements include insulators, boundary elements, scaffold/matrix attachment regions, ubiquitous chromatin opening elements, and conserved antirepressor elements. Flanking transgenes with these elements reduces the effects of heterochromatin, allowing stable transgene expression. Another option to inhibit silencing is to block the histone deacetylation using butyrate. However, homologous recombination between transfected plasmid DNA and the vector genome rarely occurs.

One way to enhance the probability of targeted integration is via enzymes such as **bacteriophage P1 Cre recombinase**, **lambda phage integrase**, or **yeast Flp recombinase** to mediate the DNA exchange between the genome and transfected plasmid. These enzymes catalyze the exchange at a high frequency if the donor and recipient DNAs are bordered by specific attachment regions. The identification of a highly active transcription site in gene targeting is crucial. If an active site is not found, the receptor site for recombination is inserted randomly, and hundreds of clones are screened for those which have been integrated into so-called good sites (Sambrook et al. 1989).

6.1.4 Selection and Optimization of Positive Clone

Since transfection efficacy is never 100%, it is highly essential to select the positive clones not only having vector but recombinant vector as well, i.e., the vector with GOI. However, in general, for efficient recombinant protein expression, it is not important whether GOI and selector genes are on the same plasmid or not. The recombinant gene and the selector gene can be present on the same vector or distinct vectors. When present on the same vector, they can be expressed using a polycistronic mRNA. To increase the chance of obtaining high-level producer cell lines, the selective gene can be driven by a weak promoter. Although this approach usually reduces the stable transfection efficiency, the cells that survive selection yield greater recombinant products.

The most popular genes for selection are **dihydrofolate reductase** (DHFR) and **glutamine synthetase** (GS). In both cases, selection occurs in the absence of appropriate metabolites (hypoxanthine and thymidine with DHFR, glutamine, and with GS, it prevents the growth of non-transformed cells). The transformants are selected using methotrexate strengthening the selection via quenched DHFR activity. This compels the cells to express more DHFR (exogenous) and consequently more recombinant protein for survival. Readers are suggested to refer to Table 3 for different marker proteins in the mammalian cells.

Similarly, most mammalian cells require glutamine. So, using a vector that contains the product gene and the GS gene allowing glutamine synthesis is always better. In such a case, the exclusive cells with the GS gene will survive. It is however necessary to include a weak promoter on the GS gene and a strong promoter on the product gene. Regarding usefulness, GS is widely used for antibody expression in NSO cells (lacking endogenous GS). GS can also be used in CHO cells using **methionine sulfoximine** to inhibit endogenous GS.

Table 3 Selective genes with their specific functions, marker genes, and corresponding actions of marker gene proteins in mammalian cells

Selective agent	Specific action	Marker protein	The action of marker protein
XyL-A	Damages DNA	Adenine deaminase	Deaminates XyL-A
Blasticidin S	Obstructs translation	Blasticidin S deaminases	Deaminates blasticidin S
Bleomycin	Breaks DNA strands	Bleomycin-binding protein	Binds bleomycin
G418 (geneticin)	Obstructs translation	Neomycin phosphotransferase	Phosphorylates G418
Histidinol	Produces cytotoxicity	Histidinol dehydrogenase	Oxidizes histidinol to histidine
Hygromycin B	Obstructs translation	Hygromycin B phosphotransferase	Phosphorylates hygromycin B
MSX	Obstructs glutamine synthesis	Glutamine synthetase	Survival of cells producing excessive glutamine synthetase
MTX	Quenches DNA synthesis	Dihydrofolate reductase	Survival of cells producing excessive dihydrofolate reductase
PALA	Obstructs translation	Cytosine deaminase	Lowers cytosine expression through its uracil conversion
Puromycin	Obstructs translation	Puromycin N-acetyltransferase	Acetylates puromycin

G418 is another important selection gene in eukaryotes, expressing the neo gene. G418 is an aminoglycoside antibiotic produced by *Micromonospora rhodorangea*. It blocks polypeptide synthesis in eukaryotic cells via irreversible binding to 80S ribosomes and subsequently disrupted proofreading capability. Resistance to G418 is conferred by the neo gene from transposon Tn5, encoding an aminoglycoside 3′-phosphotransferase (APH 3′ II3). This protein inactivates G418 by covalently modifying its amino or hydroxyl functions and concurrent inhibition of antibiotic-ribosome interactions (Sambrook et al. 1989; Zeyda et al. 1999; Wurm 2004).

6.1.5 Selection and Optimization of Cell Culture Medium

Each mammalian cell has its preference for nutrients and growth supplements which are supplemented by the cell culture medium. For small-scale laboratory culture, serum originating from the bovine animals such as fetal bovine serum (FBS) or fetal calf serum (FCS) is an important constituent. The serum not only supplies various nutrients and maintains pH but also optimizes the osmolarity of the medium and thereby the cultured cells. However, since the serum is costly, to reduce the recombinant protein production cost, serum supplements instead of serum are used. The use of serum or serum proteins also increases the chances of viral contamination and other infectious agents such as mycoplasma. Therefore, it is generally recommended to completely avoid serum use, particularly concerning large-scale culture for product commercialization. Thus, it is highly essential to standardize all serum supplementing constituents so that optimum cell growth and culture medium

osmolarity could be maintained. Moreover, most mammalian cells (except some leukocytes) are adherent in nature, the culture of which is not only difficult but poses another constraint as the recombinant proteins generated by adherent culture are much lower in quantity. Nevertheless, adherent cells such as CHO cells can be adapted to suspension culture. Here also, standardization of the culture medium and conditions is necessary so that mammalian cells grow without hassles in suspension mode (Almo and Love 2014).

6.1.6 Selection and Optimization of Cultivation Mode in Bioreactor

For producing large quantities of recombinant proteins, various large culture vessels such as bioreactors are used. Once adapted to small-scale culture, a selected high-product (protein) producing clone needs to be adapted well toward suspension-mediated growth in a serum-free medium inside a bioreactor for large-scale production of recombinant proteins. The most common cultivation modes used in bio-manufacturing are fed-batch and perfusion culture. The use of one or the other technology depends on the biochemical requirements of cultured cells concerning the maximum production of the desired protein. Cells are cultivated either attached to carriers or in suspension. HEK-293 and CHO cells are maintained as either suspension or adherent cultures, with the former being particularly favored for their ease of handling and scale-up. HEK-293 and CHO cells in suspension mode typically exhibit a doubling time of fewer than 24 h, growing to higher than five million per ml densities, depending on culture conditions. Therefore, these cells are of immense importance for mammalian recombinant protein production (Huang and McDonald 2009).

6.1.7 Purification of the Expressed Protein

Most of the recombinant proteins isolated from cultured mammalian cells are either secreted proteins or the secretion sequences added to their gene so that they can be secreted into the cell culture medium. So, the first step of purification is the collection of cell culture medium using **centrifugation**. Since the harvested/collected cell culture medium contains the secreted recombinant protein in a diluted concentration, the generated protein is thereafter concentrated using **tangential flow filtration**. This decreases the volume and thereby makes it easier to handle followed by purification using multiple methods such as **differential centrifugation**, **filtration**, **chromatography**, etc. Various fusion proteins tagged with the recombinant protein help to identify and purify the latter. These proteins include the **constant IgG domain** (the Fc region), **maltose-binding protein** (**MBP**), **small ubiquitin-like modifier** (**SUMO**), and **human serum albumin (HSA)**. All these tagged fusion proteins have been shown to improve either yield or solubility or both for the protein with which these are tagged. The Fc tag does cause artificial dimerization of fused proteins, likely to induce aggregation with certain partners. As many of these tags are large, it is often necessary to remove them after the purification of the fusion protein. The most common solution is to engineer a protease cleavage site between

the solubility tag and the partner recombinant protein (Gray 1997; Dalton and Barton 2014).

6.1.8 Quality Control and Quality Assurances

Quality control and quality assurance is the last important step before labeling and packaging recombinant mammalian proteins.

Very briefly here are the steps:

- The purified recombinant protein can be estimated via UV-visible spectroscopy using various protein concentration determination techniques such as Bradford, Lowry, bicinchoninic acid (BCA), etc.
- Then, the proteins are biochemically analyzed using SDS-PAGE, Western blot, or ELISA.
- Thereafter, the protein activity is determined using various assays involving a specific substrate (of the protein).
- The product must also be checked for possible contamination with a residual live virus (if any at all).
- After full-scale checking, a generally high concentration of purified recombinant protein is stored at −20 °C in the presence of stabilizing agents such as glycerol or polyethylene glycol (PEG) (Sissolak et al. 2017).

6.2 Major Recombinant Proteins Produced by Mammalian Cell Culture

In this section, we talk about various proteins produced *by* applying recombinant DNA technology to mammalian cells, including human cells. The tissue plasminogen activator (tPA) is the first protein produced via applying recombinant DNA technology to mammalian cell culture. Other widely used recombinant proteins produced using mammalian cell culture are urokinase, follicle-stimulating hormone (FSH), Epogen (erythropoietin), blood clotting factor VIII, Remicade (infliximab), and several monoclonal antibodies (Khan 2013).

Therefore, several recombinant proteins are produced by mammalian cell culture. Each of these proteins has tremendous importance in today's human society including in medical science.

Here is a list of some widely used recombinant proteins:

1. Tissue plasminogen activator
2. Urokinase
3. Erythropoietin
4. Follicle-stimulating hormone
5. Blood factor VIII or factor VIII antigen
6. Monoclonal antibodies

The following paragraphs briefly discuss the abovementioned proteins:

6.2.1 Tissue Plasminogen Activator

The first approved biologic from a mammalian bioprocess platform was tissue plasminogen activator (tPA), produced in 1986–1987 by *Genentech Inc*.

Here is a brief description of tPA.

Origin of Tissue Plasminogen Activator

The tissue plasminogen activator (tPA) of human origin exists in a very small quantity in human normal tissues such as blood vessels (e.g., endothelial cells), kidneys, uteri, etc. Recombinant tPA can be produced by transferring a recombinant vector containing the human tPA gene into *Escherichia coli*, yeast, or mammalian cells as host cells. Of the various mammalian cells, the culture medium of Chinese hamster ovary (CHO) cells is used for the large-scale isolation and purification of medium secreted tPA.

Structural Biochemistry of Tissue Plasminogen Activator

It is known that the tPA isolated from the culture medium can be divided into two classes, based on its molecular structure. The original tPA secreted by human melanoma cells has a single chain form, composed of 527 amino acids and some sugar chains. In the culture medium, it is converted into tPA comprising two chains, by the action of a protease within the culture medium. This protease cleaves the peptide bond between the 275th arginine and the 276th isoleucine, counted from the amino terminus of the peptide chain, finally manifesting as a two-chain form tPA, with the two strands being joined together by one disulfide linkage. Therefore, the tPA isolated in the usual method from the culture medium is a mixture of single- and double-chain tPA forms. It is recognized that these two kinds of tPA have a molecular weight of about **69,000 kDa** when measured by SDS-polyacrylamide gel electrophoresis under the nonreducing condition. However, under the reducing conditions, it is recognized that single-chain form tPA has a molecular weight of about 69,000, and double-chain tPA has two values, namely, 36,000 and 33,000. The molecule is divided into five structural domains, lasting from the N to C terminus.

These domains are as follows:

- Looped finger domain
- A growth factor domain
- Kringle 1 domain
- Kringle 2 domain
- Serine protease domain

The correct folding of tPA requires the right pairing of 17 disulfide bridges in the molecule.

Function of Tissue Plasminogen Activator

Tissue plasminogen activator (tPA) is a serine protease involved in blood clot breakdown (fibrinolysis). As an enzyme, it catalyzes the conversion of plasminogen to plasmin. Both looped finger and kringle 2 domains bind specifically to the fibrin

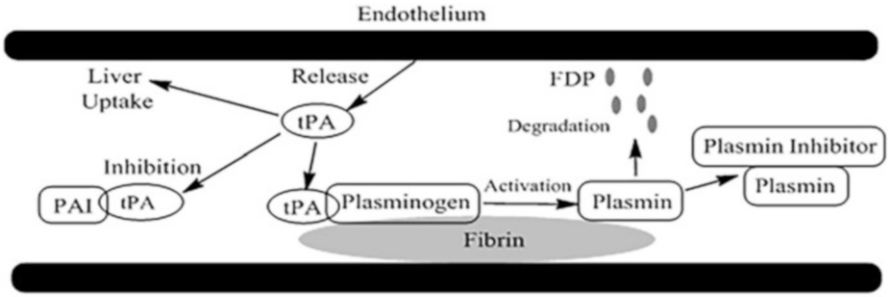

Fig. 5 The sequence of events involved in plasminogen to plasmin conversion mediated generation of fibrin degradation product (FDP)

clots, thereby accelerating tPA protein activation of bound plasminogen. Next to the kringle 2 domains are the serine protease domain that is responsible for converting plasminogen to plasmin. Plasmin is important for the homeostasis of fibrin formation and clot dissolution.

The fate of tPA within a human body can take the following three roots:

- Uptake by the liver and cleared through receptors therein.
- Inhibited by a plasminogen activator inhibitor (PAI) and subsequently cleared from the liver.
- The activation and plasminogen to plasmin conversion (for degradation) result in fibrin degradation product (FDP) (Fig. 5).

Production of Recombinant Tissue Plasminogen Activator Using Chinese Hamster Ovary Cells

The following are the steps for tPA production from the cultured CHO cells:

- Use antibody-based immunoprecipitation to isolate tPA mRNA from human melanoma cells.
- The mRNA is converted to cDNA by reverse transcriptase enzyme after which a cDNA library can be constructed.
- The resulting cDNA library is subsequently screened via sequence analysis and compared to a whole-genome library for confirming specific protein isolation and accuracy.
- Next, the cDNA is cloned into a synthetic plasmid and initially expressed in prokaryotic bacteria including Gram-negative *Escherichia coli* cells, followed by yeast cells (e.g., *Saccharomyces cerevisiae*, a single-cell eukaryote) with successful results confirmed via sequencing before attempting in mammalian CHO cells.
- The transformants are selected using methotrexate, which strengthens their selection by inhibiting DHFR activity which then compels the cells to express more DHFR (exogenous) and consequently more recombinant protein for survival.

- The highly active transformants are now placed in a bioreactor for large-scale culture.
- The tPA which is then secreted into the culture medium is collected.
- After this, the medium containing tPA is concentrated and purified (for therapeutic use) by various techniques including chromatography.
- The purified protein is then characterized.
- Recombinant tPA is commonly referred to as r-tPA and is sold under multiple brand names.
- For pharmaceutical purposes, tPA is the first pharmaceutical drug produced using mammalian cell culture, specifically CHO cell culture (Bernik and Kwaan 1969; Rijken and Collen 1981; Jones and Garnick 1990; Griffiths and Electricwala 2005).

Usefulness of Tissue Plasminogen Activator in Medical Sciences

The thrombolytic agent tPA was the first recombinant protein/drug produced by Genentech in 1988–1987 using the recombinant tPA gene in mammalian cells. Its market name is activase (alteplase). The recombinant thrombolytic tPA is safe and effective for dissolving blood clots in patients with heart diseases and thrombotic disorders. It is used to treat heart attacks, strokes, clots in the lungs, and cancer treatment.

6.2.2 Urokinase

Urokinase, also known as urokinase-type plasminogen activator (uPA), is a serine protease present in humans and other animals. It physiologically acts as a plasminogen-activating proteolytic enzyme and facilitates the dissolution of blood clots.

The following paragraphs describe the urokinase production and characterization:

Origin of Urokinase

The human urokinase enzyme was discovered, but not named, by *McFarlane and Pilling* in 1947. It is a cell-secreted protein and is therefore present in blood and urine. In the human body, urokinase is produced by many tissues and is therefore present in the extracellular matrix (ECM) of those cells and tissues. The human kidneys produce the largest amount of urokinase. It has been reported that the urokinase secreted by kidney cells is antigenically similar to the one isolated from the urine. Since the amount of urokinase in the human urine is very low, the recombinant urokinase is produced on a large scale using the cultured CHO cells or HEK-293 cells.

Structural Biochemistry of Urokinase

Urokinase is encoded in humans by the **PLAU gene**, which stands for "plasminogen activator, urokinase." Urokinase is synthesized as a zymogen (urokinase or single-chain urokinase) and is activated by proteolytic cleavage between Lys158 and Ile159. The two resulting chains are held together by a disulfide bond. There are

two configurations of this enzyme, the one with low molecular weight (LMW, 32,400) and the other being high molecular weight (HMW, 54000) urokinase. The LMW is an autocatalytic fragmentation product of the HMW and both forms are glycoproteins. The LMW form of human urokinase consists of an A chain of 2000 Daltons linked by a sulfhydryl bond to a B chain of 32,400 Daltons. The HMW urokinase is a two-chain glycoprotein containing 411 amino acids with 12 disulfide bonds. Its molecular weight is 54,000 Daltons.

This enzyme consists of the following three domains:

Serine protease domain
Kringle domain
Growth factor domain

Functions of Urokinase

Urokinase is a serine protease that activates plasminogen into plasmin by cleaving the Arg-Val linkage in the **Pro-Gly-Arg-Val** sequence of the former, which in turn degrades the fibrin clots. Thus, the primary physiological substrate of this enzyme is plasminogen, an inactive form (zymogen) of the serine protease plasmin. Activation of plasmin triggers a proteolytic cascade which, depending on the physiological environment, participates in thrombolysis or extracellular matrix degradation. This cascade is involved in vascular diseases and cancer progression.

Hence, urokinase finds its significance as an important anti-thromboembolic drug. The need for urokinase production has increased significantly in recent years, and current production levels have not kept the proportionate pace. Mammalian cells exhibit posttranslational modifications, owing to which mammalian cell lines are nowadays preferred for the production of recombinant urokinase. HEK-293 cells of human origin are a suitable host for recombinant urokinase production because they grow exceptionally well inside the bioreactor.

Production of Urokinase Using Human Embryonic Kidney Cell Line

The major source of commercially available urokinase is human urine. However, extremely low urokinase concentrations (10–15 μg/ml) in human urine create a major problem in its isolation and purification. HEK-293 cells, cultured in vitro, are a recognized source of urokinase. These cells are known to secrete biologically active and heavily glycosylated urokinase in the culture medium. Thus, since the amount of secreted urokinase in the human urine is too low, it is produced by culturing mammalian kidney cells such as HEK-293 cells. CHO cells can also be used for preferential greater urokinase production (Barlow 1976; White et al. 1966).

Production of urokinase by mammalian cell culture comprises synthesis, regulation, and secretion. Production and accumulation of this product in a hollow fiber bioreactor is a real challenge for biochemical engineers. All characterized commercial urokinase preparations contain one or both constitutive enzymatically active forms (LMW and HMW) of urokinase. The yields of this enzyme are relatively low. To enhance the urokinase production by these cells, it is required to optimize operational strategies and perform medium standardizations for cell growth and

enzyme production. Moreover, urokinase production per unit confluent area and per unit time must be increased. This potential for increased urokinase secretion by the cells is an important consideration for using cell culture-mediated urokinase production. Figure 6 outlines the salient aspects of urokinase production from a human embryonic kidney cell line.

For the separation of urokinase from the human urine, a series of concentration steps followed by conventional chromatographic separations are utilized. In one such reported work, *Hou and Zaniewski* purified urinary urokinase by an SP cation exchanger followed by a zinc-chelated affinity chromatographic cartridge reporting an 18-fold increase in urokinase-specific activity with nearly 70% yield. For separation and purification of urokinase from different mammalian cells using conditioned cell culture media, multistep chromatographic methods have been reported.

Urokinase production is a product-inhibited (feedback regulated) process. Therefore, in situ urokinase separation is required to operate a bioreactor corresponding to its maximum urokinase formation rate. Structural similarities in the known synthetic substrates and inhibitors of urokinase have enabled the formulation of suitable competitive inhibitors for affinity chromatography ligands such as **BAPA** (α-benzylsulfonyl-*p*-amino phenylalanine), amino benzamidine, homoarginine

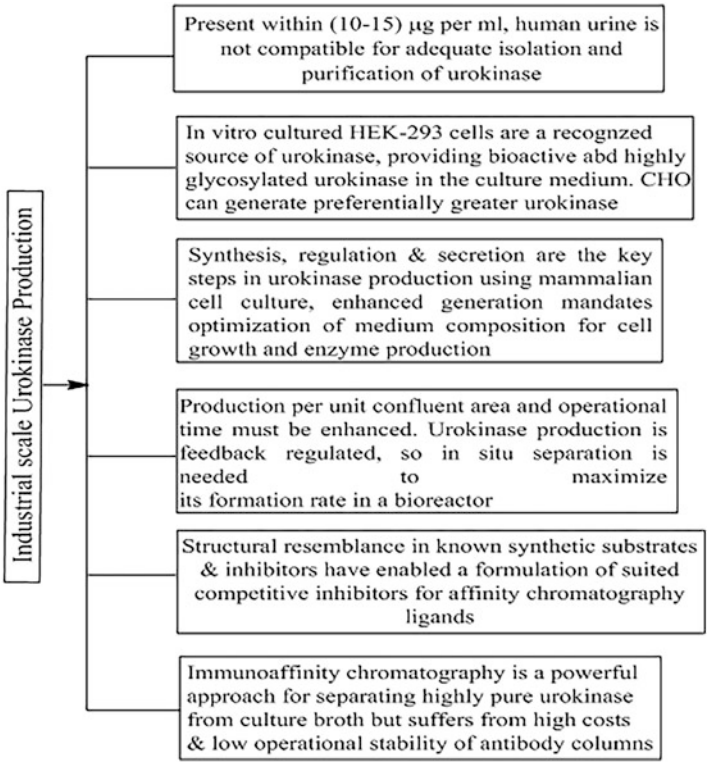

Fig. 6 Summarized aspects of urokinase production using human embryonic kidney cell line

benzyl ester, or agmatine coupled to a gel matrix. However, these adsorbents do not exhibit specificity to yield highly purified urokinase. Immunoaffinity chromatography offers a powerful approach for the separation of highly pure urokinase from the cell culture broth. However, because of the high costs and low operational stability of the antibody columns, this approach suffers from some limitations. Most of the isolation methods for urokinase use a multistep approach providing lower yields coupled with high capital and increased operating costs, thus resulting in cost and purity issues as the possible limitations in urokinase purification from crude sources.

Thus, the new developments for efficient and cost-effective urokinase separation strategies are highly desired.

Usefulness of Urokinase in Medical Sciences

Urokinase is responsible for the dissolution of clots in blood vessels. The urokinase-produced plasmin dissolves the fibrin clots in blood vessels. Perhaps due to this very reason, urokinase is intravenously administered for the treatment of thromboembolic diseases such as pulmonary embolism.

6.2.3 Erythropoietin

Erythropoietin (EPO) is a glycoprotein exclusively produced by the cells of the liver and kidney and some other mammalian body organs. It activates the hematopoietic stem cells (erythroid progenitor cells) within the bone marrow for its proliferation, differentiation, and maturation to erythrocytes or red blood cells (**RBCs**). One of the most important contributions of erythropoietin is the hemoglobin synthesis (the oxygen/carbon dioxide-carrying metalloprotein) in the RBC.

Origin of Erythropoietin

In infants, erythropoietin (**EPO**) is produced mostly in the liver, but the kidneys become the primary site of EPO synthesis shortly after birth. The adult kidney produces EPO under **hypoxic or normoxic** conditions in the arterial blood, caused by anemia. Circulating EPO binds to the receptors on the surface of erythroid progenitor cells that in turn mature into RBCs. In the 1970s, human EPO was first isolated and later purified from urine. The gene encoding EPO was cloned and several groups devised recombinant DNA methods for EPO production by the mid-1980s. Amgen Inc. holds a US patent for erythropoietin preparation using recombinant DNA technology in the CHO cell line. The recombinant protein is commercially known as **Epogen** (Davis and Arakawa 1987; Takeuchi et al. 1989; Egrie 1990).

Structural Biochemistry of Erythropoietin

Human EPO is a 30,400 Dalton molecule (30.4 kDa) containing 165 amino acids and four carbohydrate chains that incorporate sialic acid residues. There are several EPO forms, designated by Greek letters that differ only in the carbohydrate content.

Functions of Erythropoietin

EPO is a glycoprotein that serves as the primary regulator for the RBCs population in mammals. It stimulates hematopoietic stem cells or more specifically erythroid progenitor cells which differentiate into RBCs, controlling hemoglobin synthesis and red blood cell population.

Production of Recombinant Erythropoietin Using Chinese Hamster Ovary Cells

The commercially purified EPO is called Epogen. For EPO production, Chinese hamster ovary (**CHO**) cells are seeded into roller bottles that are filled to 10–30% extent with medium, and on being slowly rotated, the cells begin to adhere. The rotation assures a regular wetting of the cells and oxygen is supplied by the ample "head space in the bottle."

After a period of growth and maintenance of the culture at confluence for a few days, the product is harvested from the decanted supernatant culture medium as EPO is a secretory protein. The process can be easily scaled-up and the number of roller bottles handled in parallel determines the scale. The product is concentrated by filtration and purified by chromatographic techniques.

Product recovery is possible within the 50–200 mg/L range, providing protein in the kilogram range, on an annual basis. It is unlikely that such a process would deliver gram/liter product concentrations because the cell-to-volume ratio is much lower than in an optimized stirred-tank reactor process.

Today's Epogen process is essentially a robot-based manufacturing procedure whereby all the critical handling steps, including cell seeding, filling the bottles with cell culture medium, and harvesting of cell culture fluids/medium, are executed within air-filtered environments without human intervention. This reduces the contamination possibilities.

Usefulness of Erythropoietin in Medical Sciences

Recombinant human EPO has been effectively used to treat anemia (in general) or anemia associated with AIDS, renal failure, etc.

6.2.4 Follicle-Stimulating Hormone

Follicle-stimulating hormone **(FSH)** is a glycoprotein produced by the anterior pituitary gland that stimulates both female and male gonads for their growth and maturation. Gonadal maturation leads to the complete development of reproductive processes which completes the pubertal maturation.

Origin of Follicle-Stimulating Hormone

Follicle-stimulating hormone (**FSH**) is produced by the gonadotropic cells of the anterior pituitary gland of mammals (both male and female). The hypothalamus, the center of the autonomic nervous system, releases a hormone called follicle-stimulating hormone-releasing hormone (FSHRH). In general, FSHRH is also called a gonadotropic hormone-releasing hormone (GnRH) since it stimulates another hormone, luteinizing hormone (LH), to get released from the anterior pituitary.

After being released by the hypothalamus, FSHRH reaches the anterior pituitary through the **hypothalamic-hypophyseal portal system** and stimulates the anterior pituitary to release FSH. Of note, another name for the pituitary is **hypophysis**. Through the blood, FSH reaches the gonads (ovary/testes) and helps in ovulation (in females) and spermatogenesis (in males). The level of gonadal hormones like estrogens, progesterone, and testosterone is regulated by FSH and LH/ LTH.

Structural Biochemistry of Follicle-Stimulating Hormone

FSH is a 35.5 kDa heterodimeric glycoprotein, consisting of an alpha (α) and beta (β) peptides. The α and β chains are encoded by separate genes. FSH structure is similar to those of luteinizing hormone (**LH**), thyroid-stimulating hormone (**TSH**), and human chorionic gonadotropin (**HCG**).

The α-subunits of the glycoproteins LH, TSH, HCG, and FSH are identical and consist of 96 amino acids having five disulfide bonds, while the β-subunits vary from each other. FSH has a β-subunit of 111 amino acids (FSH-β) with six disulfide bonds, which confers its specific biologic action and is responsible for interaction with the FSH receptors. The sugar moiety of the hormone is covalently bonded to asparagine and is composed of **N-acetyl galactosamine**, **mannose**, **N-acetyl glucosamine**, **galactose**, and **sialic acid**. Of the four Ans-linked glycosylation sites in FSH, two are located on the α-subunit (Asn 52 and 78), while the other two are on the β-subunit (Asn 07 and 24). Both subunits are required for biological activity.

FSH exists in multiple charged isoforms, and the biological activity, half-life, and immunogenicity of FSH are strongly influenced by the glycosylation pattern throughout the cultivation process. Differences in constitutional carbohydrate moieties can consequently result in significant distinctions for the aforementioned characteristics.

Functions of Follicle-Stimulating Hormone

FSH is involved in the following actions:

- Stimulating the maturation of primordial germ cells and thereby regulating the development, growth, pubertal maturation, and reproductive processes of the human body (Fig. 7).
- In *males*, FSH induces **Sertoli cells** to secrete androgen-binding proteins (ABPs), regulated via inhibiting negative feedback mechanism on the anterior pituitary gland. Specifically, activation of Sertoli cells by FSH sustains spermatogenesis and stimulates inhibin B secretion.
- In *females* FSH initiates **follicular growth**, specifically affecting granulosa cells within the ovary. With the concomitant rise in inhibin B, FSH levels subsequently decline in the late follicular phase. This seems to be critical in selecting only the most advanced Graafian follicle to proceed to ovulation. Of note, ovulation is the process in which mature ovum is released from the ruptured Graafian follicle, and subsequently, the empty, ruptured Graafian follicle is converted to corpus luteum. At the end of the luteal phase, there is a slight rise in FSH, which is significant to start the next ovulatory cycle.

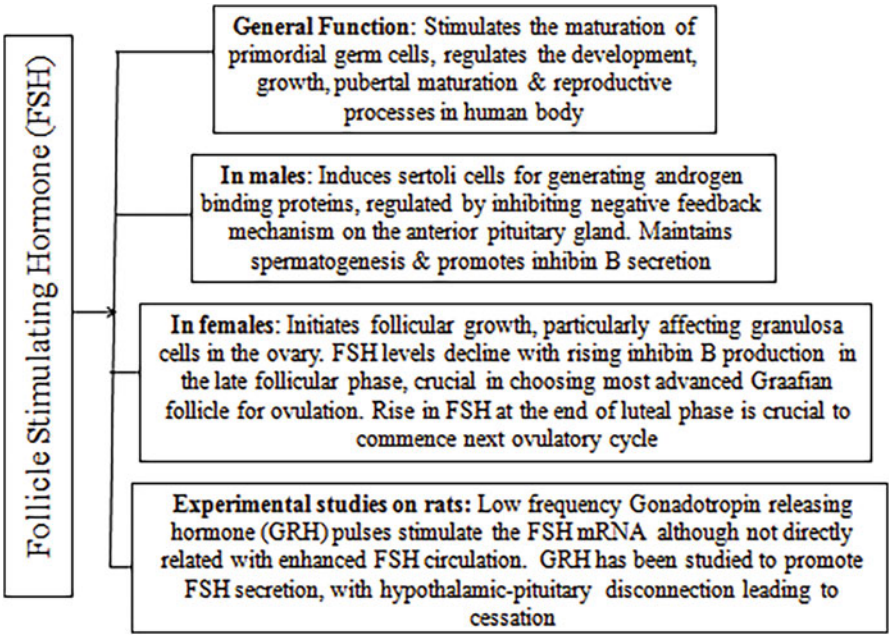

Fig. 7 Summarized functions of follicle-stimulating hormone (FSH), in males and females, and the conclusive findings of experimental studies on rats

- Experimentally in rats, low-frequency gonadotropin-releasing hormone (GnRH) pulses increase FSH mRNA but are not directly correlated with the increment in circulating FSH. GnRH has been shown to play an important role in FSH secretion, with hypothalamic-pituitary disconnection leading to its cessation. GnRH administration leads to a return of FSH secretion.
- FSH is subject to estrogenic feedback from the gonads via the hypothalamic-pituitary-gonadal axis.

Production of Recombinant Follicle-Stimulating Hormone Using Chinese Hamster Ovary Cells

The FSH present in the human urine (uFSH) is much diluted and therefore very difficult to purify. Many groups have prepared and described the actions of human recombinant FSH (rFSH) produced by transfected CHO cells. Recombinant rat FSH production by CHO cells with subsequent purification and functional characterization was also reported. Cloning and expression of **cynomolgus monkey (*Macaca fascicularis*)**, gonadotropins, luteinizing hormone, and FSH were also described.

The specific activity of rFSH is estimated at **10,000 IU/mg extent**, nearly a hundred folds greater than uFSH. The most obvious advantages of rFSH include greater purity and specificity. It has been inferred that smaller doses and a more predictable response will result in much reduced (potentially serious) ovarian hyper-

stimulation syndrome. The most dramatic disadvantage to the health provider and patient, however, is a marked increase in the product price.

Similar to most recombinant proteins, the production of recombinant human FSH (**r-hFSH**) generally follows a well-established development process involving (i) transfection of cultivated mammalian cells (e.g., CHO cells) with the human FSH gene (α-FSH and β-FSH), (ii) clonal selection and isolation of produced cell line, (iii) cell banking to supply cell substrates from the selected clone for unobstructed product manufacture, and (iv) large-scale production, involving bioreactor and purification. The development of the manufacturing process starts with pilot batches for process optimization before validation and industrial-scale production in full-scale batches with reproducible quality.

Due to their easy growth and survival adaptability in suspension culture, CHO cells are considered capable of reaching high cell densities. CHO-based processes can easily be up-scaled to more than 10,000 L bioreactors, followed by downstream processing (Howles 1996; Hakola et al. 1997).

Usefulness of Follicle-Stimulating Hormone in Medical Sciences

The utility prospects of FSH in medical sciences are as detailed ahead:

Infertility Therapy

FSH is used commonly in **infertility therapy**, mainly for ovarian hyperstimulation as part of IVF.

Anovulation Therapy

It can be used squarely well for ovulation induction and **anovulation** reversal. FSH is available as mixed with LH activity in various **menotropins** including more purified forms of urinary gonadotropins such as **Menopur** as well as without LH activity, as recombinant FSH (**Gonapure**, Gonal-F, Follistim, Follitropin alpha).

Studying the Structure and Functions of Glycoprotein Hormones

FSH is used for studying the structure and functions of glycoprotein hormones, via the creation of a genomic library in the CHO cells through cloning DHFR gene expression (Fig. 8). Since these are free from contaminating hormones, their structure and function can be studied extensively because of their substantial availability. FSH also helps to understand estrogen feedback mechanisms.

6.2.5 Blood Coagulation Factor VIII

Blood coagulation factor VIII (FVIII) is a glycoprotein in the mammalian blood coagulation cascade, the deficiency of which may lead to hemophilia A, a pathophysiological or disease condition. Hemophilia A is characterized by profuse bleeding with slow or no blood coagulation following an injury to the blood vessels. Defects in the FVIII gene result in hemophilia A, a recessive X-linked coagulation disorder having a prevalence of 1 in 5,000 males.

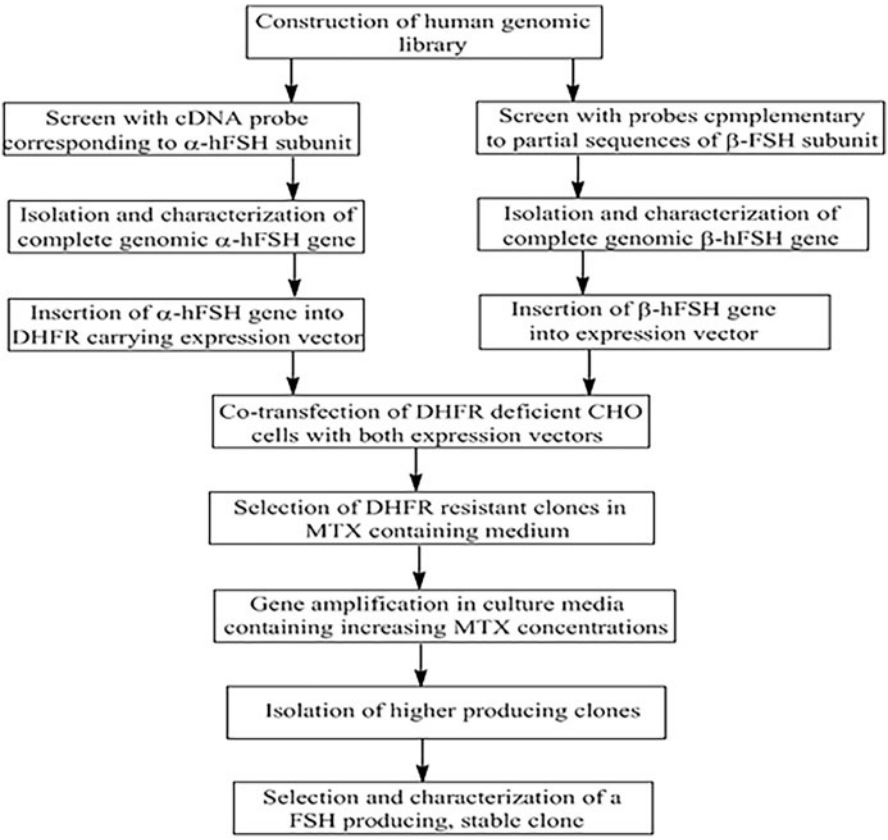

Fig. 8 Expression of hFSH in Chinese hamster ovary (CHO) cells

Origin of Blood Coagulation Factor VIII

Factor VIII is produced by the endothelial cells in the blood vessels throughout the body and by the liver sinusoidal cells. The protein is secreted in the blood and binds with von Willebrand factor, another protein released by vascular endothelial cells. This binding form of factor VIII remains **inactive** until an injury to the blood vessels separates the von Willebrand factor from it. The active coagulation factor VIII interacts with another coagulation factor, called factor IX. This interaction sets off a chain of additional chemical reactions to collectively form a blood clot. Thus, factor VIII acts as a nonenzymatic cofactor for blood coagulation.

Structural Biochemistry of Blood Coagulation Factor VIII

The secretary protein factor VIII is synthesized as a 2351-amino acid residue single-chain precursor. This chain is thereafter translocated to the endoplasmic reticulum (ER) where a 19-residue signal peptide is cleaved. The precursor protein is proteolytically processed to generate a 90 to 200 kDa sized amino-terminal-derived heavy

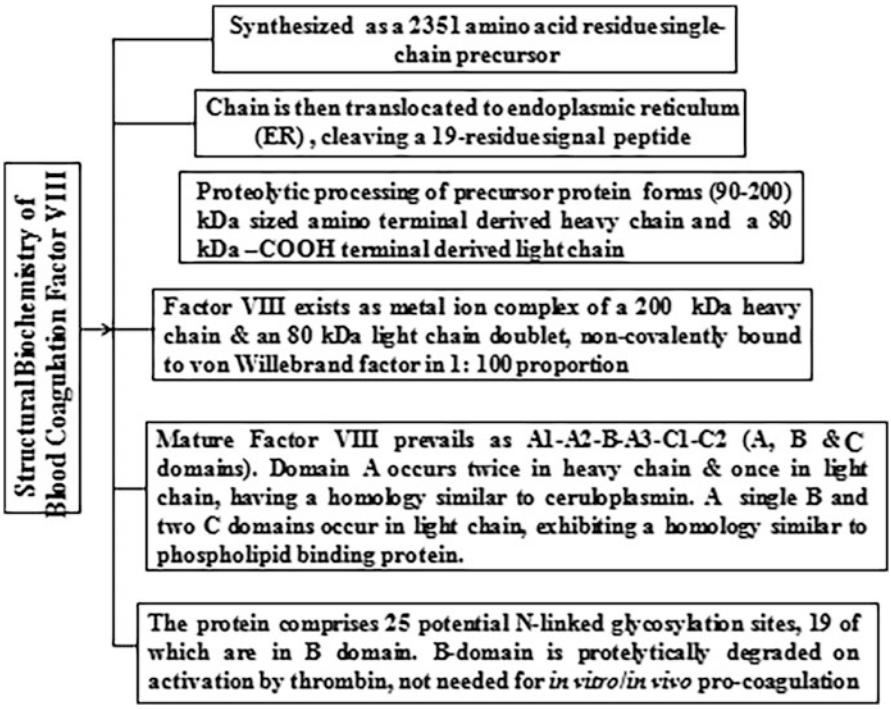

Fig. 9 Summarized structural whereabouts of blood coagulation factor VIII

chain and an 80 kDa, carboxyl-terminal-derived light chain. It is unknown whether this cleavage occurs intracellularly or in the plasma. In plasma, factor VIII exists as a metal ion complex of a 200 kDa heavy chain and an 80 kDa light chain doublet. This complex is non-covalently bound to the von Willebrand factor in 1:100 ratios.

The final mature factor VIII is composed of three domains, occurring in the order of A1-A2-B-A3-C1-C2: The A domain occurs twice in the heavy chain and once in the light chain and has homology to ceruloplasmin, a copper-binding plasma protein, a single B domain, and two C domains in the light chain having homology to phospholipid-binding proteins. The protein contains 25 potential N-linked glycosylation sites, 19 of which are in the B domain. The B domain is proteolytically released upon activation by thrombin or factor Xa and is not required for in vitro or in vivo pro-coagulation (Gitschier et al. 1984). Figure 9 summarizes the characteristic structural traits of blood coagulation factor VIII for an easier experimental follow-up.

Functions of Blood Coagulation Factor VIII

Blood coagulation factor VIII is produced in liver sinusoidal cells and endothelial cells in the blood vessels throughout the body. This protein circulates in the bloodstream in an inactive form, bound to another molecule called von Willebrand

factor, until an injury damaging blood vessels occurs. Factor VIII activates FIXa, FX, and Ca^{++} on the membrane surface and causes blood coagulation.

Blood coagulation factor VIII is proteolytically activated by thrombin, resulting from cleavage of the heavy chain in Arg372 (A1-A2 domain linkage) and Arg740 (A2-B domain linkage) amino acid sites and of the light chain in Arg1689 (B-A3 domain linkage) site. The active form of coagulation factor VIII, FVIIIa, is a trimer consisting of A1 (amino acids 1–372), A2 (amino acids 373–740), and linked A3-C1-C2 (amino acids 1690–2332) domains. The B domain does not comprise the active form of coagulation factor VIII. The function of FVIIIa in the coagulation cascade is to accelerate FX activation in the presence of FIXa, phospholipids, and calcium ions.

Production of Recombinant Blood Coagulation Factor VIII by Using Chinese Hamster Ovary Cells or Baby Hamster Kidney Cells

Owing to its large size and complexity, the expression level of recombinant FVIII is two to three orders of magnitude lower than other recombinant proteins produced in mammalian cell lines. Thus, despite the knowledge acquired over more than 25–30 years of industrial production, efficient production of recombinant coagulation factors remains a challenge for the industry, which is striving hard to improve the manufacturing operations for a timid and cost-effective treatment of patients. Currently, at least 12 recombinant FVIII and five recombinant FIX products are available in the market.

The traditional source of FVIII has donated blood plasma, which is in short supply and poses a significant risk of virus and prion transmission, even after rigorous (batch-wise) screening and multiple viral inactivation treatments. Recombinant human FVIII (rhFVIII) for **hemophilia A** treatment may be obtained from cultured mammalian cells purified to clinical grade using affinity chromatography, three or four rounds of conventional chromatography, solvent-detergent-mediated viral inactivation, and nanofiltration or heating. Marketed variants of r-hFVIII are expressed in CHO or BHK cells and are fully equivalent to the plasma-derived FVIII in replacement therapy.

The major drawback of r-hFVIII production techniques is the FVIII **low expression extent**, caused by the unusual size and structural complexity of the target protein (Andersson et al. 1986; Campos-da-Paz et al. 2008; Fantacini and Picanço-Castro 2018).

Recombinant Blood Coagulation Factor VIII Products

Several generations of blood coagulation factor VIII products are presently available on the market.

Here is the discussion on some of them:

The First-Generation Products

The first recombinant coagulation factor VIII was launched in 1992 by Genetics Institute and Baxter Healthcare Corporation, Hyland Division. **Kogenate**, made by Bayer Healthcare Pharmaceuticals, was launched a few months

later, in 1993. Both entities were developed using animal-derived proteins in the cell culture medium, having human serum albumin in the final formulation, being considered first-generation products.

The Second-Generation Products

The risk of exposure to transmissible agents (*non-enveloped viruses, hepatitis A and parvovirus B19, Creutzfeldt-Jakob agent and its variant or yet unknown agents*) has led to the development of the second-generation products, having the culture medium supplemented with human-derived proteins instead of animal-derived proteins (fetal bovine serum), wherein no albumin was added to the final formulation. In this case, new technologies were used to stabilize the FVIII, with sucrose and trehalose, for example.

The Third-Generation Products

The third-generation products involve those in which no animal or human proteins for cell cultivation and purification are used. Some studies have shown that patients treated with second-generation rFVIII products harbor a higher risk of inhibitor development compared to those administered third-generation products. FVIII inhibitor is an immunoglobulin G (IgG) produced by the patient having a high polyclonal affinity directed against the FVIII protein. Inhibitory antibodies are directed usually against the A2, A3, and C2 FVIII domains. The binding of an inhibitor in these domains results in a steric blockade of the FVIII functional epitopes.

Usefulness of Blood Coagulation Factor VIII in Medical Sciences

Hemophilia A is a blood disorder, prevailing as a sex-linked genetic disease in humans. The patients suffering from hemophilia A **lack factor VIII** which plays an important role in blood clotting. Current therapy for this disease involves the transfusion of blood factor VIII into patients.

6.2.6 Recombinant Monoclonal Antibodies

Immunoglobulins (**Ig**) or antibodies (**Ab**) are specific glycoproteins produced by the B lymphocytes and are involved in humoral immunity. An antibody carries out a dual role and is intrinsic in its structure. One Ab has two active sites, called fragment antigen-binding (**Fab**), that have the ability of independent binding with the two active sites of an antigen (**Ag**) called an epitope. An antibody also can induce an immune response. Based on the affinity of binding with the active site of an antigen (called **epitopes**), antibodies are classified either as polyclonal or monoclonal (mAbs). While a polyclonal antibody recognizes and binds more than one epitope on the Ag, the monoclonal Abs recognize and bind only one or single epitope (Trill et al. 1995; Andersen and Reilly 2004; Li et al. 2010; Chartrain, 2008; Rita Costa, 2010).

Production of Recombinant Monoclonal Antibodies in Mammalian Host Cells

The following are the steps involved in the production of recombinant antibodies in mammalian cells:

Construction of Antibody-Coding Vectors

An antibody is a complex protein comprising two identical heavy chains (**HCs**) and two identical light polypeptide chains (**LCs**). The sequences of both heavy and light chains can be commercially synthesized (e.g., by GenScript, Piscataway, NJ, USA). Each of the antibody chains should be cloned into separate expression vectors under the control of the same human cytomegalovirus (**CMV**) immediate-early enhancer and promoter. The cloned sequences are subsequently confirmed by DNA sequencing.

Co-Expression of Light and Heavy Chain Genes

Based on the heavy chain, antibodies are divided into five different classes. They are IgG (two identical γ heavy chains), IgA (two identical α heavy chains), IgM (two identical μ heavy chains), IgE (two identical lε heavy chains), and IgD (two identical δ heavy chains).

Complex interactions occur between light and heavy chains during the folding and assembly of an IgG mAb [most abundant (~80% of Ab) and smallest Ab in the human plasma]. The light chain/heavy chain peptide ratio plays an important role in the kinetics of mAb formation. The excess light chain is reported to be beneficial for higher mAb expression levels. There are also reports that light to heavy chain ratios above 1.5 result in minimal product aggregation extents. As light chain/heavy chain ratio could affect mAb assembly, it is suggested that mAb glycosylation could also vary with changing light to heavy chain proportions. It is of prime interest to mediate these mAb subunit expressions at the optimal stoichiometric ratio for a higher mAb production.

A light chain and heavy chain genes are traditionally introduced by co-transfecting on two separate vectors or via transfecting a single larger vector carrying all the required genes. Light and heavy chain peptide ratios can be varied under transient conditions while co-transfecting the genes on separate vectors by changing the relative extents of each plasmid. Controlling the ratio by this method, the transfection is usually not stable owing to a random integration process with randomly assorted gene copies and an uncontrolled site of integration. Single vectors provide better control of the ratio as these enable the integration of all genes at the same site. One possible issue that could arise with having multiple promoters nearby is the resulting transcriptional interference. This interference suppresses gene expression to varying extents depending on the site of integration. Choosing single-promoter and single-vector systems for expressing light and heavy chains have a stricter control on their mutual stoichiometries. One such system uses internal ribosome entry site (IRES) elements to express light and heavy chains.

Using Mammalian Cells for Recombinant Monoclonal Antibody Production

Mammalian cells are currently the main hosts for the commercial production of therapeutic proteins, including mAbs. African green monkey kidney (COS) cells may be appropriate if the aim is to produce mAbs in small-scale quantities, for preliminary investigation. Indeed, they have been used for transient expression of active antibodies since 1987. However, they are not the most suitable cells for large-

scale production, since they lose the production ability over time. If the aim is large-scale production, the most suited cells are the CHO cells, which have gathered increasing laboratory-scale importance. Other mammalian cells in use for mAbs production are murine lymphoid cells (NS0, SP2/0), human cells such as human embryonic kidney (HEK) cells, and PER.C6 cells.

Transfection and Generation of Stable Cell Lines

Transfection is carried out in duplicate in 6-well tissue culture plates. For each well, 2×10^6 cells are seeded and transfected with 3 μg linearized plasmid DNA, using X-tremeGENE/HD reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions.

To generate stable cell lines, CHO cells can be first transfected with LC-expressing vectors (PL or PUL) and selected with G418 (400 μg/ml) (Sigma-Aldrich). Then the resulting LC-expressing stable cells can be transfected with HC-expressing vectors (PH or PUH) before being selected with **zeocin** (500 μg/ml), 72 h post-transfection over 14 days. Stable cell lines could be generated using different antibody vector combinations: (1) CHO-HL transfected with PH and PL vectors and (2) CHO-UHUL transfected with PUH and PUL vectors.

Culture of Mammalian Cells

The selection of an expression system (cells along with suitable genetic materials) is determined by the host's capability to deliver high productivity with suitable product quality attributes. Guidelines on cell engineering for mAbs production are also reported previously. Using heterologous promoters, enhancers, and amplifiable genetic markers, the yields of antibody and antibody fragments could be enhanced.

In the development of all pharmaceutical production processes, including those of human Abs by CHO cells, decisions regarding the optimum process parameters and methods are made based on cost, time, and titer comparisons. Often, multiple scalable platforms are examined before a final process is transferred to pilot- or scale-up laboratories. Significant research and development time and capital are invested to increase yields, reduce costs, and improve the current bioreactor and bioprocess technologies.

In the batch method, all nutrients are supplied in an initial base medium. The fed-batch method adds nutrients once they are depleted. The perfusion method circulates the medium through a growing culture, allowing simultaneous removal of waste, the addition of nutrients, and product harvesting.

The application of single-use equipment is on the rise in bioprocess development owing to the reduced turn-around times and cross-contamination risks. Adding an ATF filtration device and using a solid growth-support matrix in a packed-bed vessel allow for simple cell retention with higher cell and product yields. Much greater productivity can be achieved through prolonged perfusion culture with up to 2 or 3 months of continuous antibody harvesting after the initial inoculation.

Downstream Processing of Antibodies

The cultured cells secrete Ab into the cell culture medium after which the culture medium is purified. Cell culture sample constitutes primarily the medium components such as growth factors, hormones, transferrin, secreted cytokines, etc. There may also be bacterial contamination contributing to bacterial endotoxins.

The mAb from the cell culture medium could be purified using the following steps:

Centrifugation and Filtration

The cell debris, lipids, and clotted materials are removed firstly by centrifugation and thereafter by filtration, using a 0.45 μm filter.

Ultrafiltration and Dialysis

After the removal of cell debris and clotted residual cell materials, the sample is concentrated using ultrafiltration or dialysis.

Purification of the Sample

Most of the charged impurities are usually anions such as nucleic acids and endotoxins. These can be separated using ion-exchange chromatography. The cation-exchange chromatography is used at a low enough pH over which the desired antibody binds to the column while anions flow through. Contrary to this, the anion-exchange chromatography is used at a high enough pH over which the desired antibody flows through the column while anions bind to it. Various proteins can also be separated along with the anions based on their isoelectric point (pI, pH(I), IEP). In proteins, the pI is defined as the pH, corresponding to which no charge prevails.

Large-scale mAbs purification is based on various chromatographic techniques. Protein affinity purification is used in the majority of cases in combination with at least one ion-exchange step. It is common to include at least one and sometimes more polishing steps using ion exchange, hydrophobic interaction, and/or size exclusion chromatography for Abs purification. The steps are designed to remove contaminant proteins from cells or medium (to ppm levels) and DNA (to ppb extents). Depending on the process, there may be additional specific contaminants (e.g., leached protein A) that need to be removed. In addition to contaminants, it may also be necessary to remove several undesirable derivatives of the product itself such as degradation products and aggregates.

For mammalian cell processes, one also has to take account of potential viral risks while establishing the ability of the purification pattern(s) capable of removing a range of viruses. In addition, at least two viral removal/inactivation steps are included, typically based on filtration and low pH treatment and sometimes, involving the use of solvent/detergent.

Typical yields from an antibody purification process are within the 60–80% range, depending on the number of steps. With increasing upstream concentrations, increasing attention is being paid to downstream recovery as this account for a significant proportion of the total cost and can also limit the overall plant throughput.

Antibody Quantification

Recombinant mAb concentration was measured using **sandwich ELISA** where the antibody could be characterized using Western blotting.

Usefulness of Recombinant Monoclonal Antibodies

The mAbs were first described nearly 50 years ago, by the Nobel Laureates, *Kohler* and *Milstein*. Over the years, mAbs have become invaluable assets in all spheres of our life. The mAbs are now the second-largest category of biopharmaceutical products in development and are predominantly manufactured by being cultured mammalian cells.

The following are the major area of mAbs usefulness:

Use of monoclonal antibodies in basic laboratory research
Use of monoclonal antibodies in diagnostic technologies
Use of monoclonal antibodies as therapeutic tools

Overleaf is the brief presentation of each of the above.

Use of Monoclonal Antibodies in Basic Laboratory Research

Monoclonal antibodies (mAbs) are widely used in basic laboratory techniques such as serotyping, characterization of antigens/antibodies by immunoprecipitation/immunodiffusion, immunohistochemistry, immunocytochemistry, flow cytometry, Western blotting, ELISA, and related techniques.

Radioimmunoassay; gel shift assay and gel and super-shift assay; confocal/fluorescent microscopy for detection, localization, and quantification of specific proteins/antigens; and a large number of other assays are also based on the specific binding proximity of mAbs. In these techniques, the specific mAbs bind with the specific epitope present on the Ag (the sample) and subsequently help in localization detection, quantification, and characterization of Ags/mAbs interaction.

Thus, in today's world of **immunology/cell biology/biochemistry/molecular biology/protein engineering**, mAbs are one of the basic functional probes.

Use of Monoclonal Antibodies in Diagnostic Technologies

Monoclonal antibodies have several utilities in diagnostic technology, for example, detection of the insulin level in the blood of healthy or diabetic subjects via radioimmunoassay, detection of disease-causing agents such as **HIV** in the blood (using ELISA), pregnancy test (through the urine of pregnant women: ascertaining **HCG** presence) using anti-HCG antibody, and so on.

The **immunodiagnosis** of **protozoal** and **parasitic** diseases has been significantly improved by mAb technology because the tests involving mAb as diagnostic reagents overcome the limitations of polyclonal antibodies. MAbs were found extremely useful in containing the rapid outbreak of East Coast fever (**ECF**). MAbs of diagnostic value have also been developed against *Trichomonas vaginalis*,

Leishmania donovani, *Trypanosoma congolense*, *Babesia bovis*, and many other diseases that are diagnosed today using specific mAbs.

Use of Monoclonal Antibodies as Therapeutic Tools

The use of mAbs in the treatment of various diseases (enclosed as under) is as described.

In the last 30–40 years, mAbs are predicted as useful against various cancers, chronic inflammatory diseases, and chronic infections (such as mAbs anti-HIV have shown promising results in human HIV therapy). These agents are also effective against transplantation rejection, ankylosing spondylitis, allergy, Crohn's disease, psoriasis, ulcerative colitis, and rheumatoid arthritis. The interest in this class of mAbs has increased over the years including manifold enhancement in the specific research.

Some FDA-approved antibodies used for the treatment of various cancers are alemtuzumab, bevacizumab, cetuximab, gemtuzumab ozogamicin, ipilimumab, ofatumumab, panitumumab, pembrolizumab, ranibizumab, rituximab, and trastuzumab. mAbs used against autoimmune diseases include infliximab and adalimumab and are also screened effectively in rheumatoid arthritis, Crohn's disease, ulcerative colitis, and ankylosing spondylitis through their ability to bind and inhibit TNFα, a pro-inflammatory cytokine. Basiliximab and daclizumab inhibit pro-inflammatory cytokine IL-2 on activated T cells and thereby help in preventing acute rejection of kidney transplants. Similarly, omalizumab inhibits human immunoglobulin E (IgE) and is useful in treating moderate-to-severe allergic asthma.

The commercial development of therapeutic mAbs commenced in the early 1980s, and by 1986 the first therapeutic mAb was produced. This mAb was named ortho-clone (OKT3) and was approved for use in preventing kidney transplant rejection. Since the OKT3 approval, therapeutic mAbs and antibody-related products such as Fc-fusion proteins, antibody fragments, and antibody-drug conjugates (collectively referred to as monoclonal antibody products) have emerged as the dominant product class within the biopharmaceutical market. Recombinant mAbs fulfill a large spectrum of functions spanning from research to diagnosis and treatment therapies for various diseases. Their specificity and low immunogenicity make them a significant alternative to traditional treatment regimes, increasing the accuracy of targeting specific molecules and minimizing the adverse side effects.

Recombinant antibodies have been explored as treatment agents against cancer, HIV, herpes simplex virus (HSV), and several other critical disorders. ScFv has been a part of the highly promising therapeutic approach of universal chimeric antigen receptor (uniCAR) technology, enabling authentic corrective results. The ScFv is part of the technology in the form of target modules, directing the immune response to specific cancer cells expressing the target antigen. In the case of research into HIV treatment, recombinant antibodies are rather used for their neutralizing attributes. The same holds for HSV infection wherein specific recombinant antibodies are being designed to bind with the surface heparin sulfate proteoglycan (HSP), complicating or even disabling the HSV entry into the host cell. This is a method that significantly decreases the severity of HSV infection.

Major Limiting Factors toward the Clinical Applications of Monoclonal Antibodies

Potential limiting factors toward the advancing clinical applications of mAbs include the followings:

- Immunogenicity
- Difficulty and cost of production on an adequate scale
- Unwanted biological activity due, for example, to direct effects on the cells of the immune system
- Limited binding affinity, which necessitates the injection of large antibody quantities to achieve a therapeutic effect
- Lack of direct functional action, requiring conjugation of drugs or other biologically active materials
- Limited penetration into the target tissue, especially dense, poorly vascularized tumor tissue

Overcoming the Limitations of Monoclonal Antibody

- Antibody engineering is the best option for overcoming the limitation of mAbs usefulness. Here are some way-outs through which we can overcome the shortcomings of mAbs applicability:
- Preparation from existing hybridoma genes or genes encoding for small proteins, which include the antigen-binding site but omit most of the remaining molecules including sequences responsible for the biological effects of antibodies.
- Modification to increase antigen-binding affinity (affinity maturation).
- Preparation of fusion proteins consisting of the antigen-binding site linked directly to, for example, a toxin, an enzyme, or a sequence suitable for radioisotope labeling another antibody sequence, to achieve increased or novel biological activities.
- Modification to make the sequence more humanlike and less immunogenic.
- Generation of gene libraries through derivation from human antibody genes. This avoids the immunogenicity associated with foreign protein.
- Genetic modification of mice so that they produce human antibodies followed by immunizing and hybridoma preparation in a classical way.
- Preparation of antibody fragments in bacterial culture for increased yield.

7 Mammalian Cell Culture in Cell Therapy

Cell therapy involves the delivery of living cells to a patient for the treatment of various diseases. This technique relies on the use of technologies that minimize the risk of contamination and achieve the strictly controlled, secure environment necessary for growing healthy, viable cells.

The most common cell therapy is blood transfusion involving the transfusion of red blood cells, white blood cells, and platelets from a donor. Another common cell

therapy is the transplantation of hematopoietic stem cells to create the bone marrow which has been performed for over 40 years.

While there is no formal classification of cell therapy based on the origin, the process may be called autologous or allogenic (cell therapy). The cells used in cell therapy can be classified by their potential to transform into different cell types. For instance, pluripotent cells can transform into any cell in the body and multipotent cells can transform into other cells, but their repertoire is more limited than pluripotent cells. Differentiated or primary cells are of a fixed type (Fischbach et al. 2013).

The following paragraphs chronologically describe autologous and allogeneic cell therapy and then various stem cells used for cell therapy:

7.1 Autologous Cell Therapy

In this process, the cells are derived from the patient and modified in the laboratory (usually genetically) before being re-administered back to the patient. Since the cells originated from the recipient's own body, there will be no chances of rejection. The patient-specific nature of autologous cell therapies can make large-scale production extremely challenging because a separate batch needs to be produced for each patient. Companies often struggle to scale up manufacturing as a necessary practice, in clinically and commercially viable ways.

In 1997, the US Food and Drug Administration (**FDA**) approved a product consisting of autologous cultured chondrocytes (**Carticel**, Genzyme Biosurgery). Derived from the in vitro culture of a patient's normal femoral articular cartilage, this product showed clinical benefits in developing hyaline cartilage that is lost in the event of acute or recurring trauma. However, adverse reactions, most commonly hypertrophic tissue development, have been reported in both intra- and postoperative circumstances.

7.2 Allogeneic Cell Therapy

In this process, the cells are derived from another individual and reveal a certain resemblance with the potential new host, similar to blood transfusion. Some companies are exploring the use of allogeneic cell therapies, deriving treatments or doses for multiple patients from the cells of a single donor. Since single donor cells are administered to several patients, manufacturing may be efficient and cost-saving.

However, the use of cell therapies (cells derived from one donor and administered to a different recipient) involves a greater risk of immune rejection. As a consequence, cells for allogeneic therapies must be extensively cultured and cryopreserved for long-term storage. This prolonged culture increases the risk of cell transformation (e.g., tumorigenicity), and often the frozen product requires further manipulation before administration.

7.2.1 Basic Reasons for Cell Therapy

Cell therapy involves the transfer of cells having a relevant function into a patient.

Cell therapy is adopted in the following conditions:

1. Diseases that are caused by a gene mutation
2. Diseases that are resistant to conventional therapy for which there is no effective treatment
3. Cases where current therapy involves long-term administration of an expensive therapeutic agent or an invasive procedure

Many non-communicable human diseases such as cancers, rheumatoid arthritis, diabetes, Parkinson's disease, Alzheimer's disease, etc. can be treated by cell therapy or gene therapy. Present-day cancer cell therapy focuses on eliminating the cancer cells, blocking tumor vascularization, and boosting the immune response to tumor antigens.

The following paragraphs describe the cell therapy procedure and various cells being used for cell therapy:

7.2.2 Cells Utilized in Cell Therapy

In general, various stem cells are used for cell therapy. Since a separate chapter (Chap. 12) is dedicated to stem cell culture, here we briefly discuss various stem cells.

Embryonic stem cells
Induced pluripotent stem cells
Nuclear transfer of embryonic cells
Parthenogenetic embryonic stem cells
Hematopoietic stem cells
Mesenchymal stem cells
Neural stem cells
Epithelial stem cells
Immune cell therapy

Here is a very brief discussion of various stem cells utilized in cell therapy:

Embryonic Stem Cells

Embryonic stem cells (**ESCs**) are derived from embryos. Generally, the embryos used to isolate stem cells are unused embryos generated from in vitro fertilization (**IVF**) for assisted reproduction. As ESCs are pluripotent, they retain the ability to self-renew and form any cell in the body. ESCs have the advantage of versatility due to their pluripotency, but the use of embryos in the development of therapeutic strategies raises some ethical concerns. In addition, stem cell lines generated from embryos are not genetically matched to the patient, owing to which there is an increased likelihood of transplanted cell rejection by the patient's immune system.

Induced Pluripotent Stem Cells

Induced pluripotent stem cell (IPSC) is a differentiated adult (somatic) cell, such as a skin cell, and is reprogrammed to return to a pluripotent state. These cells offer the advantage of pluripotency. IPSCs may be derived from a patient and thus avoid the problem of immune rejection. IPSCs are produced by transforming the adult cell with a cocktail of genes usually delivered via a viral vector. While the efficiency of the process has been greatly improved since its inception, the relatively low rate of reprogramming remains a concern. Another concern is that IPSCs are derived from adult cells and are, therefore, "older" than ESCs as evidenced by enhanced programmed cell death, lower rate of DNA damage repair, and increased incidence of point mutations.

Nuclear Transfer of Embryonic Cells

Nuclear transferred embryonic stem cells **(ntESCs)** are pluripotent cells produced by transferring the nucleus from an adult cell obtained from a patient to an oocyte obtained from a donor. The process of transferring the nucleus reprograms the egg cell to pluripotency. As with iPSCs, the derived cells match the nuclear genome of the patient and are unlikely to be rejected by the body. However, the major advantage of this technique is that the resulting ntESCs carry the nuclear DNA of the patient alongside mitochondria from the donor, making this technique particularly appropriate for diseases where the mitochondria are damaged or dysfunctional. A drawback of ntESCs is the cumbersome generation process, requiring a donor oocyte. This technique has only been successful in lower mammals and for higher animals, including wherever human research is going on.

Pathogenetic Embryonic Stem Cells

For the production of parthenogenetic embryonic stem cells **(pES)**, the oocyte is treated with chemicals that induce embryo generation without the addition of sperm (parthenogenesis), and ESCs are harvested from the developing embryo. This technique generates ESCs that are genetically identical to the female patient. However, this method is in the early stages of development, and it is not yet known if cells and tissues derived from parthenogenesis are capable of normal development.

Hematopoietic Stem Cells

Hematopoietic stem cells **(HSCs)** are multipotent blood stem cells that give rise to all types of blood cells. HSCs can be found in adult bone marrow, peripheral blood, and umbilical cord.

Mesenchymal Stem Cells

Mesenchymal stem cells **(MSCs)** are multipotent cells present in multiple tissues including the umbilical cord, bone marrow, and fat tissue. MSCs give rise to bone, cartilage, muscle, and adipocytes (fat cells) which promote the marrow adipose tissue.

Neural Stem Cells

Adult neural stem cells **(NSCs)** are present in small numbers within defined regions of the mammalian brain. These multipotent cells replenish neurons and supporting cells of the brain. However, adult neural stem cells cannot be obtained from patients due to their residing in the brain. Therefore, neural stem cells used for cell therapies are obtained from **iPSCs or ESCs**.

Epithelial Stem Cells

Epithelial stem cells (EpSCs) are those which form the surfaces and linings of the body including the epidermis and the gastrointestinal tract lining. Multipotent epithelial stem cells are found in these areas along with unipolar stem cells that only differentiate into one kind of cells. Epithelial stem cells have been successfully used to regenerate the corneal epithelium.

Immune Cell Therapy

Cells that rapidly reproduce in the body such as immune cells, other blood cells, or skin cells can usually do so ex vivo given the right conditions. This allows differentiated, adult immune cells to be used for cell therapy. These cells can be removed from the body, isolated from a mixed cell population, modified, and then expanded before being reintroduced into the body. A recently developed cell therapy involves the transfer of adult self-renewing T lymphocytes that are genetically modified to augment their immune potency. Various other immunological cells such as dendritic cells, cytotoxic T cells, etc. can also be isolated from the cancer patients before being cultured in the laboratory and thereafter, injected (adoptive transfer) into the same cancer patient to increase the potential immunity and destruct the cancer cells.

7.2.3 Phases of Cell Therapy

Cell therapy can be described as the following four phases:

Discovery
Process optimization
Production
Therapeutic delivery

Here is a brief presentation of the above phases:

Discovery

In this phase, the major working domain involves new product characterization. Functional assays and cellular and molecular profiling are useful tools for this phase.

Process Optimization

The process optimization stage involves quantifying the relationship between culture parameters and cell output. The rational design of experiments and high-throughput screening using micro-culture platforms can be used to generate empirical cell-based

models, which can be thereafter integrated with molecular profiling technologies to develop more mechanistic, molecular-based models.

Production

Considerations in the production phase include the scale-up strategy and quality control.

Therapeutic Delivery

The final phase is the therapeutic delivery of the cell product. It should be noted that the development phase's feedback on one another during process optimization affects the biological discoveries which could be made. The design space gradually becomes better defined during the production phase. Specific issues associated with cell transplantation include biological or donor-to-donor variability, microbiological contamination, immunological responses to alloantigen, and tumorigenicity of the transplanted cells. The delivery of a cell therapy product will depend on its effectiveness in a clinical setting and the ability for its viable manufacture from a translational viewpoint.

7.2.4 The Modern Challenges of Cell Therapy

While cell therapies exhibit significant promise in bringing innovative and much-needed treatments to patients, several key issues currently limit their broader adoption.

Ahead are some challenges of cell therapy:

- Safety concerns over the administration of the live cells to the patients, uncertainties over the regulatory aspects of these therapies, unknown downstream effects, and the need for sufficient investment to fully commercialize the research are frontline challenges that must be overcome to accelerate the furtherance of cell therapies.
- Owing to the huge variability potential within treatments, an additional challenge in cell therapy is the requirement of robust manufacturing processes that consistently deliver safe and effective products. Cell therapy manufacturing processes can range from the simple expansion of autologous cells that would be administered back to patients to the complex genetic manipulation of allogeneic cells that could be stored and banked for the treatment of multiple patients. The regulations surrounding cell therapies must therefore be sufficiently broad to cover a wide range of therapeutic processes while still safeguarding product safety and quality.
- From a manufacturing point of view, autologous cell therapies can be more challenging to carry out than allogeneic cell therapies as each dose is a single batch and is derived from a different source. This presents problems for cell culture, as cells from different patients are more likely to respond in distinct ways, and thereby, the source material will contain a variety of cells with widely dissimilar growth and differentiation capabilities.

- Additionally, if the cells are stored together, there is an increased contamination risk across batches. Contrary to autologous cell cultures, allogeneic cell therapy manufacturing processes are less prone to cross-contamination as they are usually derived from a single large batch. However, other manufacturing challenges do pose a constraint.
- Maintaining product consistency throughout the different stages of development is often a difficult task as many variables can affect cell growth. As such, developers of cell therapy products must consistently monitor their processes and adopt the best cell culture methods to ensure that products are manufactured with the desired critical quality attributes.

8 Mammalian Cell Culture in Gene Therapy

Gene therapy involves the transfer of genetic material (e.g., a gene), usually in a carrier or vector, and the uptake of the gene within the appropriate cells of the body. In the broadest sense, gene therapy involves the introduction, removal, or change in the content of a person's genetic code to treat or cure a disease that cannot be treated by traditional therapy. The transferred genetic material significantly alters the production of a single or a group of proteins, produced by the specific somatic cells. Gene therapy can be used to reduce the expression levels of a disease-causing version of a protein, resulting in increased production of disease-fighting or generating new/modified proteins (Wu et al. 2002).

Gene therapy can be divided into the following few types:

Gene addition
Gene correction
Gene silencing
Reprogramming
Cell elimination

Ahead is a brief discussion of these gene therapy variations:

8.1 Gene Addition

This process involves the insertion of a new gene copy into the target cells to augment a higher protein expression. Most often, a modified virus such as an **adeno-associated virus (AAV)** is used to carry the gene into the cells. Therapies based on gene addition are being developed to treat many diseases, including **adenosine deaminase severe combined immunodeficiency (ADA-SCID)**, **congenital blindness**, **hemophilia A**, **Leber Congenital Amaurosis**, **lysosomal storage diseases**, **X-linked chronic granulomatous disease**, and many others.

8.2 Gene Correction

This process can be achieved by modifying a part of a gene using recently developed gene-editing technology (e.g., **CRISPR/cas9, TALEN, or ZFN**) to remove repeated or faulty gene elements or to replace a damaged or dysfunctional DNA region. The goal of gene correction is to produce a protein that functions in a normal manner rather than contributing to a disease's development. It may be possible to use gene correction in the treatment of a wide range of diseases; recent experimental work has used gene editing to extract HIV from the genome of affected laboratory mice and to excise the expanded region responsible for Huntington's disease, from the human genes.

8.3 Gene Silencing

This process prevents the production of a specific protein by targeting messenger RNA (mRNA) for degradation so that no protein is produced. The mRNA exists in a single-stranded form in human and animal cells, whereas some RNA viruses have double-stranded RNA (dsRNA). Human and animal cells recognize dsRNA as being viral in origin and destroy it to prevent its spread. Gene silencing uses small RNA sequences to bind unique sequences in the target mRNA, making it double-stranded. This triggers mRNA destruction using the cellular machinery that destroys viral RNA. Gene silencing is an appropriate gene therapy-mediated disease treatment wherever too much of a protein is produced. For example, too much tumor necrosis factor-alpha (TNFα) is often observed in the afflicted joints of rheumatoid arthritis patients. As TNFα is needed in small amounts by the rest of the body, gene silencing is used to reduce TNFα expression extents in the affected tissues.

8.4 Reprogramming

This process involves adding one or more genes to the specific cells with the intent to change their characteristics. This technique is particularly powerful in tissues where multiple cell types exist and the disease is caused by the impaired functioning of one kind of cell. For example, type I diabetes occurs because many of the insulin-producing islet cells (β cells) of the pancreas are damaged. At the same time, the cells of the pancreas that produce digestive enzymes are not damaged. Reprogramming these cells so that they start producing insulin offers a novice treatment for type I diabetic patients.

8.5 Cell Elimination

This strategy is typically used to destroy malignant (cancerous) tumor cells but can also be used to target the overgrowth of benign (noncancerous) tumor cells. Tumor

cells can be eliminated via the introduction of "suicide genes," which enter the tumor cells and release a prodrug that induces cell death in those cells. Viruses can be engineered to develop an affinity for tumor cells. These oncotropic viruses can carry therapeutic genes to increase toxicity within the tumor cells, stimulating the immune system to attack the tumor or inhibit the growth of blood vessels supplying nutrients to the tumor.

8.6 Potential Level of Gene Therapy

Three potential levels of gene therapy are known concerning their application to treat genetic diseases (Fig. 10).

Level 1: Substitutional gene therapy is a transient, non-heritable replacement or augmentation of a working product for a null gene product, one that is typically knocked out by a mutation. The repeated use of recombinant human insulin by insulin-dependent diabetics as a replacement for genetically defective insulin and glucocerebrosidase in the case of **Gaucher disease** is an example of successful substitutional gene therapy.

Level 2: Somatic cell gene therapy is a long-term, non-heritable modification of somatic cell genomes to cure or prevent deleterious conditions, without modifying the gametes or germ-line cells. Typically, body cells are removed from an (genetically modified or engineered) individual before being placed back into the same

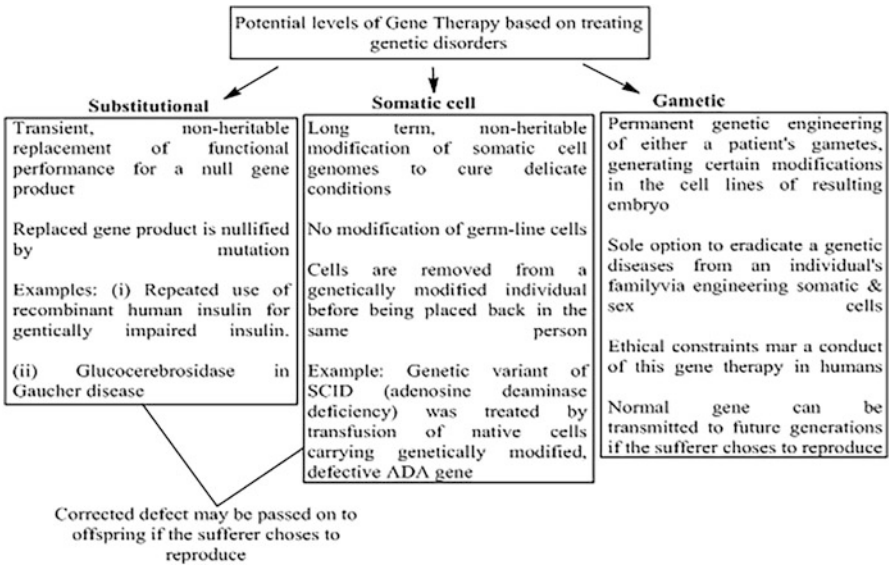

Fig. 10 Summary of gene therapy variations, distinguished via inheritance of corrected gene from the parent to the next generation

individual. The first successful application of somatic gene therapy in humans occurred in the well-known case of *Ashanti de Silva*, a 4-year-old girl affected with a genetic form of SCID called adenosine deaminase deficiency, who, on September 14, 1990, at 12:52 pm, successfully received a transfusion of her cells carrying her genetically modified, defective ADA gene. Similarly, an aerosol system delivering a genetically engineered CFTR product to cystic fibrosis patients has also been developed. These first two levels of gene therapy substitute or repair genes in a patient's body cells only wherein the sex cells quite uncharacteristically remain unaffected. If the patient then chooses to reproduce, the children may be affected with the same condition as that of the parent.

Level 3: Gametic gene therapy (also called germ-line or germ-cell gene therapy) is the practice of permanent genetic engineering of either a patient's gametes or a fertilized pre-morula zygote after which certain alterations are perpetuated in all the cell lines of a resulting embryo and the normal gene may be transmitted to future generations if the patient later chooses to reproduce. To date, **gametic gene therapy** has never been attempted in humans. Gametic gene therapy is the only form of gene therapy that can potentially eliminate a genetic disease from an individual family line by engineering both body (somatic) and sex/gonadal cells so that subsequent reproduction results in unaffected offspring. Ethical issues seriously limit the conduct of gametic gene therapy in humans.

8.7 Process of Gene Delivery to the Target Cells

Scientists and clinicians mainly use both viral and nonviral vector methods to carry genetic material into the targeted cells.

Here is a brief discussion about them:

8.8 Viral Vector Methods

Viruses have an innate ability to invade normal cells in a potential host. Viral vectors for gene therapy are modified to utilize the cell entering ability of viruses after disabling the viral capability to further divide. Different viruses have been engineered to function as gene therapy vectors. In the case of adeno-associated virus (**AAV**) and retrovirus/lentivirus vectors, the gene(s) of interest and control signals replace all or most of the essential viral genes in the vector to prevent replication of the viral vector.

For oncolytic viruses, such as adenovirus and herpes simplex virus, fewer viral genes are replaced, and the virus is still able to replicate in a restricted number of varying cell types. The different viral vectors preferentially enter a subset of different tissues, express genes at different levels, and interact with the immune system distinctively.

8.9 Nonviral Vector Methods

Nonviral vector methods such as electroporation, passive delivery, and ballistic delivery are also used for gene therapy. Simple strands of naked DNA or RNA can be pushed into cells using high-voltage electroporation. This is a common technique used in the lab. Naked DNA or RNA may also be taken up by target cells using a normal cellular process called endocytosis after it is added to the medium surrounding the cells. Finally, (sheer) mechanical force facilitates the introduction of genetic material with an instrument called a "gene gun."

All these techniques are already briefly discussed earlier. Genetic material can be packaged into artificially created liposomes (sacs of fluid surrounded by a fatty membrane) that are more easily taken up by the cells than naked DNA/RNA. Different types of liposomes are being developed to preferentially bind the specific tissues. Recent work has utilized a membrane vesicle subtype that is endogenously produced and released by cells (extracellular vesicles or "exosomes") to carry small RNA sequences into specific tissues.

8.10 Combination of Cell and Gene Therapy

Importantly, gene therapy can be combined with cell therapy protocols as discussed previously. In such attempts, cells are collected from the patient or matched donor before being purified and expanded in vitro. Scientists and clinicians then deliver the gene to the cells using one of the earlier described methods. The cells that express the therapeutic gene are thereafter, readministered to the patient.

8.11 Risks Associated with Cell and Gene Therapy

Risks of any medical treatment depend on the exact composition of the therapeutic agent and its selective route of administration (intravenous, intradermal, or surgical). Risks include the outcomes limiting the successful conduct of gene or cell therapy in line with the expectations, possibly prolonging or worsening symptoms, or complicating the conditions with manifold adverse effects.

- The administration of genetic material may induce a strong immune response to the protein in the case the replaced proteins are associated with genetic diseases. Ultimately, an aggravated immune response is noted, leading to normal proteins or cells being attacked, as in autoimmune diseases.
- On the other hand, in cancer or viral/fungal/bacterial infections, the immune response may be below par (or lower than normal); otherwise, the targeted cell or microorganisms may develop resistance to the therapy.
- With the current generation of vectors in clinical trials, there is no optimized mechanism to "turn off" gene expressions, and the chances of developing unwanted effects are quite high.

- With retroviral or lentiviral vectors, integration of the genetic material into the patient's DNA may occur next to a gene involved in cell growth regulation, and the insertion may induce a tumor over time via "insertional mutagenesis."
- High doses of some viruses can be **toxic** to some individuals or specific tissues, especially if the individuals are immunologically compromised.
- Gene therapy evaluation is generally done after birth. There is little data on what effects this therapeutic approach might have on embryos owing to which pregnant women are usually excluded from clinical trials.

8.12 Usefulness of Gene and Cell Therapy in Medical Sciences

Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has emerged as an asset for doctors for the timely diagnosis of fetal disorders. The damaged cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting, and other molecular techniques.

The usefulness of gene therapy can be divided into the overleaf sections based on the specific genetic disorders they cause:

Gene therapy against various cancers
Gene therapy against muscular disorders
Gene therapy against eye/vision disorders
Gene therapy against cardiovascular disorders
Gene therapy against neurological disorders
Gene therapy against bone disorders

Here are some examples of FDA-approved gene therapy against various diseases:

8.12.1 Gene Therapy against Various Cancers

- In August 2017, the US FDA approved the use of Kymriah (Novartis) for the treatment of specific instances of B-cell acute lymphoblastic leukemia. This chimeric antigen receptor **(CAR) T-cell** therapy modifies the patient's immune cells (T cells) to engineer their aggressive countering of leukemia cells. Clinical trials are currently underway for additional uses of Kymriah in adult patients and other forms of cancers. More information is expected to be made available in the near future.
- In October 2017, *Yescarta* (axicabtagene ciloleucel, Axi-Cel, Kite Pharma, Inc), another CAR-based T-cell therapy, was approved by the US FDA for the treatment of non-Hodgkin lymphoma, adult sufferers with a history of at least two failed systemic therapies.
- **Yescarta** (Kite Pharma Inc./Gilead) became the first US FDA-approved CAR T-cell therapy for use in adult patients, in October 2017. The drug was approved

to treat adults with relapsed or refractory large B-cell lymphoma and was the second-ever approved mediator for CAR T-cell therapy.

- **Gendicine** gene therapy drug, harboring the Tp53 gene, has been developed to treat head and neck squamous cell carcinoma (**HNSCC**). This recombinant adenovirus was developed by Shenzhen Sibiono GeneTech and was approved by China Food and Drug Administration (CFDA) on October 16, 2003. The drug found its way to the commercial market in 2004. In gendicine (drug), the E1 region of human serotype 5 adenovirus (Ad5) is replaced by human wild-type Tp53. The Tp53 expression in cancer cells stimulates antitumor attributes by initiating apoptotic pathways, suppressing DNA repair and antiapoptotic events, and seizing the survival pathways. The vector is produced in HEK293 cells by co-transfection of the Tp53 expression cassette shuttle vector with an Ad5 genome recombinant plasmid. The cassette contains the Rous sarcoma virus (RSV) promoter, the wild-type human Tp53 gene, and a bovine poly-A signal. Upon intra-tumor injection at a concentration of $\mathbf{1 \times 10^{12}}$ viral vector particles per vial, gendicine binds to the coxsackie virus-adenovirus receptor, entering the tumor cells via receptor-mediated endocytosis, expressing ectopic Tp53 gene. The most common side effect of gendicine is self-limiting fever (of 37.5 °C to 39.5 °C), normally occurring 2–4 h after administration, and lasting for approximately 2–6 h.
- As the first oncolytic virus approved by **CFDA**, recombinant human adenovirus type 5 (rAd5-H101) was commercially marketed under the brand name **Oncorine**, in November 2005. The drug was manufactured by Shanghai Sunway Biotech and was initially licensed for the treatment of patients with terminal stage refractory nasopharyngeal cancer in combination with chemotherapy, following phase III of the clinical trial.
- **Rexin-G** is a retroviral vehicle harboring a cytocidal cyclin G1 construct, considered the world's first tumor-targeting injectable gene therapy vector, approved by FDA for metastatic pancreatic cancer. The drug was officially approved by the Philippine FDA in December 2007 resulting in the progression of clinical studies to phase III trials, in the USA.
- **Pegaptanib** was developed by EyeTech Pharmaceuticals and Pfizer Inc. with the brand name Macugen. It is a polynucleotide aptamer targeting vascular endothelial growth factor (VEGF165 isoform) for neovascular age-related macular degeneration (AMD) treatment. Pegaptanib was the first anti-angiogenic drug approved by the US FDA in December 2004 and was the only therapy for AMD treatment. It is also the first therapeutic aptamer with RNA structure, achieving FDA market approval. AMD is the most common cause of severe vision loss and blindness among aged individuals in the developed world. It is characterized by deterioration of the central region of the retina. Abnormal blood vessel growth accounts for 90% of severe vision losses. It has been suggested that VEGF plays a prominent role in the growth and permeability of new vessels in AMD. Anti-VEGF agents are used as molecular therapies which attempt to block angiogenesis as well as vessel permeability. Pegaptanib is a **28-mer RNA oligonucleotide** covalently linked to two-branched 20-kD polyethylene glycol chains. It

specifically binds to the VEGF165 isoform at the heparin-binding site, thus preventing the heparin binding to VEGF receptors located on the vascular endothelial cells' surface. VEGF165 has been implicated in pathological ocular neovascularization via enhanced vascular permeability and inflammation. The recommended dose is 0.3 mg/90 μl pegaptanib, administered once every 6 weeks via intravitreal injection into the eye. Based on preclinical data, Pegaptanib is metabolized by endo- and exonucleases and is not influenced by the cytochrome P450 system. In two clinical trials involving 1186 participants, the efficacy of pegaptanib was determined by the ability of patients to lose less than 15 letters of visual acuity from baseline without dose-response monitoring. The result demonstrated pegaptanib as an effective AMD therapy. Moreover, a clinical trial assessed the side effects and the efficacy of pegaptanib in the treatment of 23 participants suffering from neovascular AMD with a previous history of arterial thromboembolic events (ATEs). Pegaptanib administration did not reveal any systemic or ocular side effects nor did it leads to any recurrent ATEs.

- **Imlygic or talimogene laherparepvec** is a genetically manipulated oncolytic herpes simplex virus (HSV) type 1, developed against multiple solid tumors such as unresectable cutaneous, subcutaneous, and nodal lesions of melanoma. Imlygic was created by BioVex Inc. under the brand name, OncoVEX$^{GM\text{-}CSF}$. The drug was approved by the US FDA in October 2015 for targeting melanoma. It was subsequently approved in Europe and Australia in 2016.

8.12.2 Gene Therapy against Muscular Disorders

- In May 2019, the FDA approved Zolgensma for spinal muscular **atrophy** treatment in less than 2-year-old children.
- **Eteplirsen** was developed by Sarepta Therapeutics under the trade name, Exondys 51. This drug is a **30-mer phosphorodiamidate morpholino oligomer (PMO)** designed to cause exon 51 (of dystrophin gene) depletion. Expression of functional dystrophin (a protein) in patients with **Duchenne muscular dystrophy (DMD)** having mutated DMD gene is amenable by skipping exon 51. The studied group of patients involved nearly 13% of all DMD cases, making exon 51 a suitable gene therapy target. In September 2016, the FDA approved Exondys 5 in a hurried procedure based on the dystrophin production in skeletal muscle as noticed in some cases, upon being treated with the drug.
- **Nusinersen**, commercialized under the name Spinraza by Biogen, was the first-ever medication approved for **spinal muscular atrophy (SMA)** treatment. Nusinersen was approved by the DA in December 2016 and by **European Medicines Agency (EMA)** in May 2017.

8.12.3 Gene Therapy against Eye/Vision Disorders

- The first virally delivered gene therapy to be approved for clinical usage in the USA, **Luxturna** (Spark Therapeutics), was approved in December 2017 by the FDA. Luxturna is a one-time gene therapy treatment used to improve vision in patients with established genetic vision loss due to Leber congenital amaurosis or retinitis pigmentosa, both being inherited retinal diseases.

- **Vitravene**, also called **fomivirsen**, is an antisense oligonucleotide (ASO) designed as a therapeutic strategy for cytomegalovirus (**CMV**) retinitis in HIV-positive patients, having no viable option for CMV retinitis treatment. Fomivirsen is the first-ever gene-silencing antisense therapy approved for marketing by the FDA. This drug was developed through a collaboration between Isis Pharmaceuticals and Novartis Ophthalmics and was approved by FDA in August 1998 and a year later by EMA/EMEA to treat cytomegalovirus retinitis.

8.12.4 Gene Therapy against Cardiovascular Disorders

- In 2010, the Human Stem Cells Institute of Russia developed **Neovasculgen (PI-VEGF165)**, a plasmid DNA encoding VEGF 165 under the control of a CMV promoter for atherosclerotic, peripheral arterial disease (PAD) treatment. The drug was listed in Vital and Essential Drugs (EUVED) of the Russian Ministry of Health in 2012 and was then distributed in the Russian market.
- **Mipomersen**, with a market name of **Kynamro**, emerged useful as an adjunct therapy for homozygous familial hypercholesterolemia (**HoFH**). Mipomersen was developed by Ionis Pharmaceuticals as a novel ASO inhibitor for the HoFH cure. It was rejected by EMA in 2012 due to cardiovascular and liver adverse effects. However, in January 2013, the FDA approved its marketing as an orphan drug for HoFH management.
- **Alipogene tiparvovec**, marketed as **Glybera**, is the gene therapy drug for **lipoprotein lipase deficiency (LPLD)** treatment. It was developed by Amsterdam Molecular Therapeutics (AMT) in April 2012. In October 2012, the European Commission (EC) approved UniQure as the marketing authorization of Glybera for treating LPLD. Glybera is the first licensed gene therapy product for an inherited disorder in Europe.

8.12.5 Gene Therapy against Neurological Disorders

With the brand name Onpattro, patisiran is the only FDA-approved RNA interference (RNAi) drug targeting polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (**hATTR**). The FDA approved this targeted RNA-based drug on August 10, 2018. Alnylam Pharmaceuticals, Inc. (NASDAQ), the leading RNAi therapeutics company, developed this lipid complex drug to treat familial **amyloid polyneuropathy (FAP)** in adults.

8.12.6 Gene Therapy against Bone Disorders

Invossa (TissueGene-C) has completed phase III trials in the USA and attained marketing approval in Korea by Kolon TissueGene as a first-in-class cell-mediated gene therapy strategy for symptomatic and persistent knee osteoarthritis (OA) treatment. It contains a 3:1 mixture of non-transformed and retrovirally transduced allogeneic chondrocytes that upregulate transforming growth factor β1 (TGFβ1) expression.

8.13 Gene Therapy against Other Diseases

- **Defibrotide**, commercially known as **Defitelio**, is manufactured by Jazz Pharmaceuticals plc. Defitelio is a DNA derivative anticoagulant used for patients with hepatic sinusoidal obstruction syndrome/**veno-occlusive disease** (**SOS/VOD**) with renal or pulmonary dysfunction following the cytoreductive treatment before hematopoietic stem cell transplantation (**HSCT**). The efficacy data from 528 investigated hepatic VOD participants exhibiting renal or pulmonary dysfunction following HSCT supported approval of defibrotide by the FDA in March 2016. It was also evaluated and approved by EMA in May 2017.
- Recently, AveX, a drugmaker owned by pharmaceutical giant Novartis, developed **onasemnogene abeparvovec** with the brand name **Zolgensma**. It is the most recent authorized gene therapy drug by the FDA (May 2019). It was previously well-known with the compound name **AVXS-101**. Zolgensma is a proprietary gene therapy strategy for curing pediatric patients below 2 years, who have mutations in both alleles of the *SMN1* gene. Zolgensma has been designed to render a healthy copy of the SMN gene to arrest the disease progression by maintaining normal SMN gene expression with a single, one-time intravenous infusion.

9 Mammalian Cell Culture in Cell Fusion Technology

In cell fusion technology, two cells of either the same or different lineages use together to form a single cell.

Based on the nucleus, the fused cells can be divided into two groups:

Synkaryon
Heterokaryon

Here is a brief description of synkaryon and heterokaryo.

9.1 Synkaryon

When the fusion of two cells gives rise to only one cell having a single nucleus with 4 N, it is called a synkaryon. In the case of synkaryon, chromosomes are ultimately lost or resorted.

9.2 Heterokaryon

When the fusion of two cells gives rise to only one cell having two nuclei with **2 N + 2 N** chromosomal distribution, it is called heterokaryon.

Cell fusion technology has widespread applications in molecular cell biology, such as the following:

- Production of hybridoma for antibody generation
- Study of the malignancy tissues
- Gene mapping
- Study of the controlling gene expression, differentiation, and viral application

9.3 Methods of Cell Fusion

In the 1960s, hybrid cells were successfully produced from mixed cultures of two different mouse cell lines, in France for the first time. The procedure involved induction of cells growing in the culture using some viruses such as the **Sendai virus** to fuse and form hybrids. This virus induces two different cells to form heterokaryons, at first. During mitosis, the heterokaryon chromosomes move toward the two poles and later on the fuse to form hybrids. It is important to remove the surface carbohydrates to facilitate cell fusion. Besides Sendai virus-dependent cell fusion, some other fusion methods include **electrical cell fusion**, **polyethylene glycol cell fusion**, and **thermoplasmonics-induced cell fusion.**

The four methods of cell fusion are as below:

1. Electrically facilitated cell fusion
2. Polyethylene glycol aided cell fusion
3. Sendai virus-induced cell fusion
4. Thermoplasmonics-induced cell fusion

Ahead is a brief discussion of the above four methods of cell fusion:

9.3.1 Electrically Facilitated Cell Fusion

The process was discovered in 1978 by *Zimmermann*, who has initiated ongoing development ever since.

The Principle of Electrically Facilitated Cell Fusion

First, the cells are brought into very close contact via **dielectrophoresis**. Unlike electrophoresis, in which direct current is applied to move molecules, dielectrophoresis uses high-frequency alternating current. In particles such as living cells, dipoles are induced, driving cell alignment in a manner resembling a string of pearls that are in very close contact with each other.

A very short high-voltage pulse is then applied, which causes permeation of the cell membrane and the subsequent combining, thereafter resulting in cell fusion. To stabilize the process, an alternating voltage is then applied for a brief duration. The resulting formation is described as a heterokaryon because, despite the fusion of the outer cell membrane, two or more cell nuclei still prevail. The cell nuclei also fuse at

a later stage within the cell. In most cases, this results in a drastic reduction in the chromosomal number in the nucleus.

By definition, electrically assisted fusion is a double-conditioned process:

1. Close physical contact between cells has to be established.
2. Cell membranes have to be brought into a fusogenic state.

Physical contact between cells can be achieved in several ways, though the most widely used is dielectrophoresis, where cells are aligned in pearl chains using alternating electric fields. Dielectrophoresis is most frequently used especially in hybridoma technology and the production of cell vaccines since it enables establishing contacts between suspended cells.

The second condition for electrically assisted cell fusion involves the achieving of membrane fusogenic state via electric pulse application resulting in structural rearrangement of the lipid bilayer.

It is generally accepted that the transmembrane voltage induced on the cell membrane during exposure to high electric fields reduces the energy barrier for the formation of hydrophilic pores in the lipid bilayer, although other explanations are also plausible. The phenomenon is termed electroporation and is related to the experimental witness of the dramatic increase in membrane permeability. At the same time, membrane fusogenicity correlates with electroporation. Both the extent of electroporation and fusion yield can be controlled by the amplitude, duration, and a number of the applied pulses. Increasing any of the mentioned pulse parameters leads to enhanced membrane electroporation and consequently a greater number of fused cells. However, parameters of the electric pulses must be carefully chosen to ensure that electroporation is reversible, i.e., cells survive. A deviation from this leads to irreversible cell electroporation, thereby reducing cell survival as well as consequent viable fused cell yield.

At a given electric field, strength of the extent of membrane electroporation further depends on the cell size. One of the major advantages of electrofusion is the possibility of optimizing electroporation conditions for each cell line on an individual basis. Unfortunately, there is a substantial challenge in fusing cell lines that differ considerably in their size. Electric pulses that are usually used for electrofusion range from 10 to 100 μs, ensuring that cell membranes become fully charged amidst exposure to electric pulse. Under such conditions, the induced transmembrane potential difference is proportional to the cell radius, implying that small cells are electroporated (i.e., brought into a fusogenic state) at higher electric field strengths. Applying pulses that effectively electroporated small cells thus inevitably leads to excessive electroporation and consequently the death of large fusion partner cells. An example where a difference in cell size hinders the optimization of pulse parameters is the hybridoma technology since B lymphocytes (approximate radius of human B lymphocytes corresponds to 3.85 ± 0.35 μm) are considerably smaller than myeloma cells (approximate radii of human and mouse NS1 cells correspond to 5.25 ± 0.25 μm and 7.75 ± 0.25 μm, respectively).

The efficiency of electrofusion depends on the following parameters:

Size of the cells to be fused
Pulse parameters
Composition of the fusion medium
Osmolarity of the fusion medium
Temperature
Post pulse cell incubation
Other factors

However, despite many efforts for improving electrofusion of cells having dissimilar sizes, higher susceptibility of larger cells to electric pulses remained one of the obstacles, and the number of viable hybridomas obtained concerning the number of input B lymphocytes remained only ~1% or less.

It is an essential step in some of the most innovative modern biology methods. This method begins when two cells are brought into contact by dielectrophoresis. Dielectrophoresis uses a high-frequency alternating current, unlike electrophoresis in which a direct current is applied. Once the cells are brought together, a pulsed voltage is applied. The pulse voltage causes the cell membrane to permeate and subsequent combining of the membranes results in cell fusion. After this, an alternative voltage is applied for a brief duration to stabilize the process. The result of this is that the cytoplasm of two cells gets mixed and the cell membrane has completely fused. All that remains separate is the nuclei, which fuse at a later stage within the cell, producing a heterokaryon cell, as a result.

Exposure of cells or liposomes to a brief pulse of a strong electrical field can result in a reversible breakdown of the outer membrane. Such breakdown causes an enhanced plasmalemma permeability which however reseals after a short incubation (i.e., till when the original impermeability is restored). Two or more cells in contact can be made to fuse by this process, provided that the contact is close enough and that the electric field pulse is short enough not to damage the cells. Methods of achieving this contact by electrical and magnetic fields are well-demonstrated. The magnetic method does not require a manifested low conductivity of media (used earlier). Other possible modifications of this flexible technique are also described and are used to understand the modification of the technique and its subsequent application to membrane research, medicine, and plant breeding.

9.3.2 Polyethylene Glycol Aided Cell Fusion

Polyethylene glycol (PEG)-mediated cell fusion is a simple and efficient technique used widely for the production of somatic cell hybrids and nuclear transfer in mammalian cloning. Fusion can be performed between adherent and suspension cells. Either whole cells or microcells can be used as donors to fuse with recipient cells. Microcell fusion is particularly useful in the transfer of a single or a limited number of chromosomes between various cells.

PEG is capable of fusing a wide variety of cells, including interspecific and interkingdom cell types. PEG exhibits a very low degree of cytotoxicity; it is only toxic at high concentrations (greater than 50%) and on a prolonged incubation (typically minutes for most cell lines). PEG is a water-soluble fusogen unlike

nonpolar or amphipathic chemical **fusogens**, such as fatty acids, retinol, and lysolecithin which act by entering the bilayer. Thus, it is relatively easy to remove PEG from the cell medium by washing. The use of PEG has several additional advantages over other fusogens: reproducibility in fusion capabilities, high fusion efficiency, inexpensiveness, easier availability, and ease of handling. The attainment of cell fusion by PEG involves its action as a dehydrating agent, attaining the fusion of not only plasma membranes but also intracellular membranes. This leads to cell fusion since PEG induces cell agglutination and cell-to-cell contact. Though this regime of cell fusion is the most widely used, it still has downfalls. Oftentimes, PEG can cause uncontrollable fusion of multiple cells, leading to the appearance of giant polykaryons. Also, standard PEG cell fusion is poorly reproducible and different cells have various fusion susceptibilities. This kind of cell fusion is widely used for producing somatic cell hybrids and nuclear transfer in mammalian cloning. Thus, PEG-mediated cell fusion is a simple and efficient technique used widely for the production of somatic cell hybrids and nuclear transfer in mammalian cloning. Using this method, one can successfully introduce mammalian mini-chromosomes into a variety of vertebrate cells.

9.3.3 Sendai Virus-Induced Cell Fusion

- Cell fusion by the Sendai virus occurs in four distinct temperature-regulated steps. During the first stage (lasting no longer than 10 min), viral adsorption takes place and the adsorbed virus is thereafter inhibited by viral antibodies.
- The second stage is typical 20 min and comprises a pH-dependent process, wherein the addition of viral antiserum can inhibit ultimate fusion.
- In the third step, the antibody-refractory stage and viral envelope constituents remain detectable on the cell surface.
- During the fourth stage, cell fusion becomes evident with a concurrent disappearance of HA neuraminidase and fusion factor. The first and second stages are the only two pH-dependent steps.

9.3.4 Thermoplasmonics-Induced Cell Fusion

Thermoplasmonics is based on a near-infrared (**NIR**) laser and a plasmonic nanoparticle treatment. The laser which typically acts as an optical trap is used to heat the nanoscopic plasmonic particle to very high and extremely local elevated temperatures. Optical trapping of such a nano-heater at the interface between two membrane vesicles or two cells leads to immediate fusion, inferred via simultaneous content and lipid mixing. Advantages include full flexibility of which cells to fuse and the conduct of fusion in any buffer condition, unlike the salt-affected electro-formation.

10 Conclusions

The primary focus of this chapter was to discuss the usefulness of mammalian cells such as CHO, BHK, HEK, COS, NSO, HT1080, and PER-C6 and their products concerning a thorough understanding of various physiological and

pathophysiological events. The topics primarily discussed include (i) use of mammalian cells as a model system for physiological and pathophysiological studies; (ii) use of mammalian cells for experimental drug/toxin research; (iii) usefulness of mammalian cell culture in vaccine production, particularly against pathogenic viruses; (iv) usefulness of mammalian cell culture for various recombinant protein production such as tissue plasminogen activator, urokinase, follicle-stimulating hormone, blood clotting factor VIII, and erythropoietin; (v) mammalian cell culture in cell therapy; and finally (vi) mammalian cell culture in gene therapy. This chapter will enhance readers' understanding of the usefulness of cells, their culture, and the corresponding products in cell biology, physiology/pathophysiology (e.g., cancers), biotechnology, pharmacology, and medical sciences.

11 Cross-References

- ▶ Experimental Mammalian Cell Culture-Based Assays
- ▶ Large-Scale Culture of Mammalian Cells for Various Industrial Purposes
- ▶ Organ, Histotypic and Organotypic Culture, and Tissue Engineering

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Biosensors' Utility in Mammalian Cell Culturing

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https://doi.org/10.1007/978-981-19-1731-8_18-2

Abstract

Over the past three decades, sensors have swiftly emerged as the fundamental requirement for ascertaining the progress of molecular events. Biosensors prevail as a formidable member under this umbrella, wherein biological entities or engineered biomaterials are used as a probe to primarily screen biological interactions. The potential of biomaterials as molecular sensing moieties draws inspiration from their renewable nature, along with the possibility of being functionalized in diverse regimes. The distinguishing aspect of biomaterials as sensing probes prevails in terms of their specificity through which their involvement in the screened interactions is minimized. A blossoming aspect in this regard pertains to the development of robust shape and size-dependent nanomaterials (NMs) whose integration with biomaterials imparts a further precision enhancement to the screening of molecular events. The architecture of noncovalent self-assembly with van der Waals forces as stabilizing factors manifests as the key prospect of biological sensing. The development of nanobiomaterials has therefore catalyzed a robust screening of biological interactions as the NMs with enhanced surface area exhibit tremendous functionalization potential. This chapter is, therefore, focused on the utility of mammalian cells or the products thereof as biosensing probes with the most emergent applications of disease diagnosis and handheld devices.

Keywords

Biosensors · Quantum confinement · Biomaterials · Surface plasmons · Shape and size dependent properties · Dynamic light scattering · Luminescence · Quantum dots · Nanocrystals · Functionalization · Surface engineering · Miniaturization

1 Introduction

Biological materials endow smart responsive behavior, characterized by their dynamic patterned structures attuned to specialized functioning and incorporation of natural bioactive compounds. These remarkably efficient architectural designs of biomaterials have been optimized ever since the origin of life on earth, thanks to stimulus-sensitive transport and mechanical properties thereof. Such attributes propel biomaterials as attractive candidates for flexible electronic sensing technologies. The most important criterion relates to the effect of the biological activity of biological materials, which aids in superseding the limited bioavailability and restricted morphology of materials involved in the fabrication of traditional flexible electronic devices. Mounting requirement for monitoring the nativity of biological interactions has necessitated the need for robust biomaterials, capable of specified biological efficacy, along with the modulation of screened interaction into a readable and modifiable electronic impulse. Conventional materials cease to be suitable here owing to their concurrent nonresponsiveness to biological stimulus and concurrent interactions. Conventional sensing makes it extensively preoccupied with inorganic/organic compound-based entities, such as carbon materials, metal oxide semiconductors, and polymers. Despite significant improvement in material attributes of these entities, their intrinsic mechanical properties, high cost, and nonbiocompatibility arrest the biological stimulus detection feasibility of these materials. Biological materials are complex actuators and possess manifold attributes suitable in this regard, such as extraordinary topography, adequate in situ tailorability (capable of being adapted to a particular function) of chemical composition, and splendid mechanical properties. Apart from structural features, readily available natural biological materials have additional advantages in being renewable, cost-effective, water-soluble, biodegradable, self-adhesive, biocompatible, nontoxic, antimicrobial, and having good adsorption. A unique characteristic of natural biological materials is their natural optimized state, manifested since the inception of evolution, harnessing acquaintance for specified requirements. For instance, our immune system comprises several nano-biointerfaces having cancer cell recognition ability. Similarly, lotus leaves have superhydrophobic surfaces enabling self-cleaning properties, along with low water adhesion. Likewise, insect-eating plants are conferred with an immensely accurate sensing mechanism for enclosing their prey. Thereby, the master class on the natural phenomenon has plenty to ponder new insights from nature for deigning further novel materials. Some other prominent natural biological entities include their natural 3D structure (like in honeycomb and natural cellular materials), imparting tunable elastic modulus to provide exceptional mechanical flexibility, such that the material can effectively and congruently assort to manifold curved and dynamic surfaces and interfaces, such as human organs and biological organism. The feasibility of affording a wide variety of functionalities (i.e., recognition, selective adsorption, and sensing) comprising several functional groups, such as –OH, –COOH, $–NH_2$, and $–NH_3$ groups, confers the properties that are seldom ever noticed in synthetic and man-made materials.

This chapter focuses on the above-briefed and related attributes of biomaterials capable of propelling them for sensing applications. Emphasis has been laid on the detection of redundant and immunologically critical disorders such as cancers, diabetes, cardiovascular complications, and several others.

2 Suitability/Principles of Biological Materials as Sensing Agents

Mechanical interactions cement a highly fundamental locus from biological concern. Mechanical forces resulting from chemical interactions elucidate the equilibrated distribution of kinetics (motility) and potential energy exchange (adhesion) on the cellular scale. These factors subsequently emerge as decisive factors for regulating molecular scale transport and affinity. Sensing through biological entities offers manifold significant opportunities to quantify the binding forces (BFs), displacements, and mass changes emanating out from cellular and subcellular events. In course of monitoring chemical interactions, concurrent specifications urgently press for monitoring of dynamic structural simulations as a result of varying interactive stoichiometries.

Natural biological materials have viciously emerged as frontrunners in such applications owing to the remarkable receptiveness of their physicochemical and biological characteristics. A thorough understanding of such attributes can revolutionize the applications of biological materials by enhancing a practical understanding of the amalgamated possibilities from different research fields. Biological materials endow splendid performance attributes through natural selection, enabling perfection in adapting to their surrounding environments. Therefore, we discuss the fundamental properties of biological materials with an emphasis on the latest advances suitable for sensing applications. A mandatory assumption here is that not all properties of biological materials are entirely well-defined. The discussion broadly focuses on the interaction of biological materials with the stimulus of varying nature, which would ideally enable sensing of the stimulus in as much unaltered form as possible (mandating no chemical interactions between the biological sensing agent and the stimulus to be detected). Owing to a legal binding on not including more than 50 references, readers are suggested to refer to the 2017 review article by Wang and colleagues for the details of referred studies included in this section. The literature source focuses on the electronic properties of biological materials and is published in *Chemical Society Reviews*.

2.1 Organized 3D Biological Assemblies

- Structure-driven properties are known for multiple specialized functions in natural creations and have inspired several technological innovations. Biological materials are inherently complex composites exhibiting structural diversity spanning several orders of magnitude in length. The exceptional characteristics are the

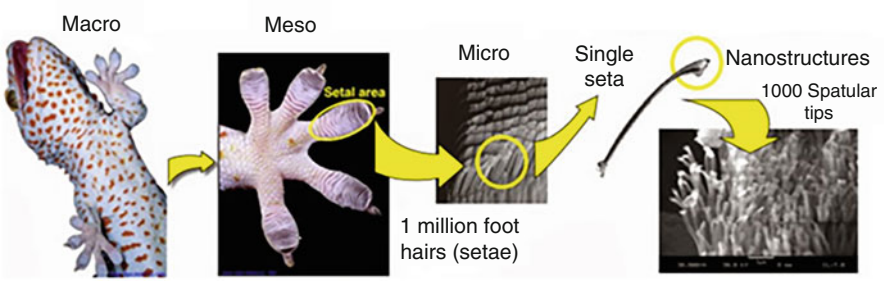

Fig. 1 Pictorial view of the gecko foot hair structures, where each seta is divided into hundreds of split ends, spatulas. Such provisions facilitate infinitesimal intermolecular forces as van der Waals forces to provide the needed adhesion. (Figure included after taking inputs from http://bioimicryreport.blogspot.com/2014/02/gecko-feet.html)

outcomes of functional adaptation of their structure at distinct hierarchical platforms. In particular, organized 3D structures exhibit extraordinary replication of their constitutional design ingredients such as small fibers, tubes, open and closed foams, and plates.

- These structures span into eight distinct categories, having been thoroughly investigated by Meyers and subsequently reviewed by Espinosa and colleagues. The classification includes fibrous, helical, gradient, layered, tubular, cellular, suture, and overlapping structures. The most common entity herein is wood (fundamentally a composite) comprised of open spaces (lumens) and interconnected cells (tracheids), having a natural cellular structure. The varied arrangement and interior structure of spaces are responsible for the different porosity of various wood species. For instance, softwood species are characterized by uniform and closely packed rectangular cells, facilitating infiltration of solutions through the porous structure, whereas a large number of closely packed (1–100) μm ranged tubular cells, including growth ring boundaries, earlywood vessels, latewood vessels, fibers, rays, and axial parenchyma cells in marginal bands, have been traced in hardwood species (such as poplar) (Wang et al. 2017).
- Tissues from several different plants and animals are richly bestowed by their multiple architectural traits for desired transport and mechanical requirements. These structural characteristics comprise the spatial and geometrically specific distribution with 3D topography and the explicit arrangement of building blocks. Hierarchical entities from nature (e.g., seashells, bones, wood, bamboo, exoskeletons) possess numerous pores in their structures. Their high specific surface areas, extraordinary permeability, storage capability, and mass transfer properties make them structurally unique. Together, these features are highly useful in several inter- and cross-disciplinary applications, ranging from catalysis, adsorption, separation processes, sensors, energy security, confronting low food grain output, and ensuring their high shelf-life.
- Two illustrations of such capability quite amicably elucidate the multifunctional abilities of self-hierarchical structures. A gecko foot, for instance, is composed of

a large number of well-configured microsized hairs with a 500 mm^{-2} approximate density. The assembly portrays a unique assorted structure with the hairs comprised of numerous smaller nanoscale structures (carrying 100–1000 single hair in density). The multiscale structure hierarchy in gecko feet lends them manifold functions and abilities, including extraordinary adhesiveness, and super-hydrophobic and self-cleaning nature (Fig. 1).

- Erstwhile structures exhibiting hierarchical arrangements include seashells with two layers of distinct microstructures. These layers can be divided into peripheral prismatic calcite layers while the outer layer has a greater susceptibility toward brittle failures and the soft inner layer of nacreous aragonite can withstand comparatively greater inelastic deformation. Apart from this, there is a third layer consisting of self-assembled mesoscale that attributes to a significant dissipation of mechanical energy. The inner layer in this assembly consists of multiple microscopic aragonite polygonal tablets with (5–8) μm diameter, 400 μm thickness, and (20–30) nm organic material constituting a thick region (Wang et al. 2017).

2.2 Biological Activity

Biological materials in the relevant size range and corresponding activity possess several properties on par with those of synthetic materials. Biological material research has emerged as a most productive research domain in nanobiotechnology through the remarkable interlinking of biological, physical, and chemical sciences for developing advanced tools having the potential to revolutionize wearable and flexible electronics. The characteristic properties responsible for the expansion of biological materials into diversified areas are as follows:

- Remarkable mechanical strength and flexibility for obtaining wearable electronic-grade biological materials.
- Altered colored appearances of biologically suited materials are capable of forming specified platforms to make sensors suited for optical transduction. Such materials can revolutionize the diversified requirements of anti-forgery devices, optical gas sensors, and biosensors for the detection of cancer and air pollution.
- With remarkable water-resistant ability contributing to self-cleansing and a meager water adhesion, the biological materials provide several benefits for use in printing, optical transduction, ultra-precision sensors, and energy storage batteries.
- Robust adhesive properties of biological materials over several 3D surfaces manifest a smart system for further conjugating bonding encompassed with alluring functionalities.
- Nearly all biological materials are antimicrobial, exhibiting suitability for making implantable antimicrobial materials in medical devices (such as artificial electronic skin).

The following sections describe the abovementioned properties of biological entities about their sensing amicability.

2.3 Mechanical Properties

The major mechanical features conferring suitability of sensing applications to biological materials include flexibility, stiffness, strength, and fracture toughness, imparting them robustness for applications in multiple domains.

The traits of flexibility, toughness, and tensile strength imbibe biological materials toward fascinating wearable green electronics. Some critical aspects of these are as follows:

- Well-ordered 3D hierarchical structures having nano- to microscale dimensionality. The mechanical properties are inherently defined by the microstructure, wherein distinctive character allows biological materials to function as active units capable of meeting specific demands. Meyers and coworkers discuss these structural design prospects leading to diverse mechanical properties of biological materials. For instance, chitosan exoskeletons consist of well-organized repetitive hierarchical structures with remarkable mechanical flexibility, comprising several discrete levels (Fig. 2). These exoskeletons can be further divided into a large number of smaller fragments. The combination of overlapping segments herein depicts an improved mechanical response arising out of overall flexibility and spontaneous internal movements (refer Wang et al. 2017 for discussed studies).
- The overlapping structures confer consistent modified responses, such as bending, deformation, and drape, the last one serving as a motivational breakthrough

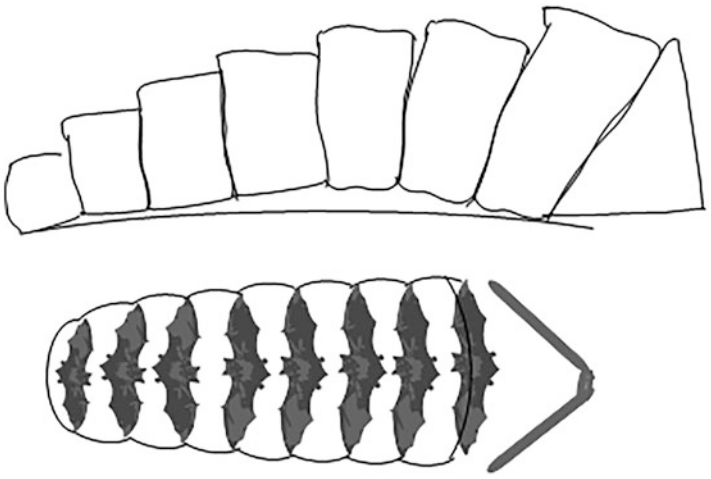

Fig. 2 Partly covered structures from chitin (Polyplacophora) represent a range of biological hierarchies for improved biological sensing

for several researchers toward designing novel biological composites equipped with splendid mechanical flexibility for developing flexible green electronics. Such overlapping structures could be easily located in shark skin, fish scales, butterfly wings, seahorse tails, and pangolin plates, and decisively impart efficient joined flexibility and working potential.

- Highly efficient flexible electronic devices could be easily available as such from animals, such as natural spider silk (having micron-scale diameter) is a highly apt example of an assorted structure comprising numerous aligned fibers. Fibrous structures are also very finely fitted entities for strength, high tension, and effectively low to null compression. Molecular configurations of natural silk present another befitting framework for dynamic response channeling. At low-stress levels, natural-grade silk responds via entropic folding of amorphous strands (uncoiling and straightening of protein strands). Contrary to this, at high pressure, the major interactions are weak hydrogen bonds so that the crystalline domains can sustain the load.
- The credit for illustrating the intricate stress–strain correlation of fibrous assemblies under physical stimulus goes to Meyer's group. Its quantitative version is as depicted in Eq. 1:

$$\sigma = K_1 \varepsilon^{n+1} + H(\varepsilon_c) E(\varepsilon - \varepsilon_c) \tag{1}$$

where σ is the stress, K_1 is a material parameter, ε is the strain, and n varies as per the chosen material. The constant as an exponent, n being 1, relates to the mechanical response of collagen support, and H is the Heaviside function and is usually activated at the beginning of the second regime ($\varepsilon = \varepsilon_c$, the strain corresponding to entirely extended geometry of fibers; refer Wang et al. 2017 for discussed studies).

- So, natural silk as a remarkable fibrous material exhibits high tensile strength or unidirectional stiffness apart from having robust mechanical properties and insulating nature. These attributes make natural silk a highly useful entity for being used as biocompatible passive support and packaging material for flexible electronic devices. Readers are suggested to refer the rigorous contributions of Meyer's group, where the authors have described the quantitative profile of mechanical properties of several other biological materials, along with their structural advantages.

2.4 Structural Coloring

The colored features of living organisms generated on account of iridescence, pigmentation, or superposition of two distinct colors are in themselves a kind of boost for sensing-based applications. Among these several modulations, structural color is a distinctive natural trait exhibited by the interaction and transport of ordered structures with light. The hierarchical structural arrangement in biological structures

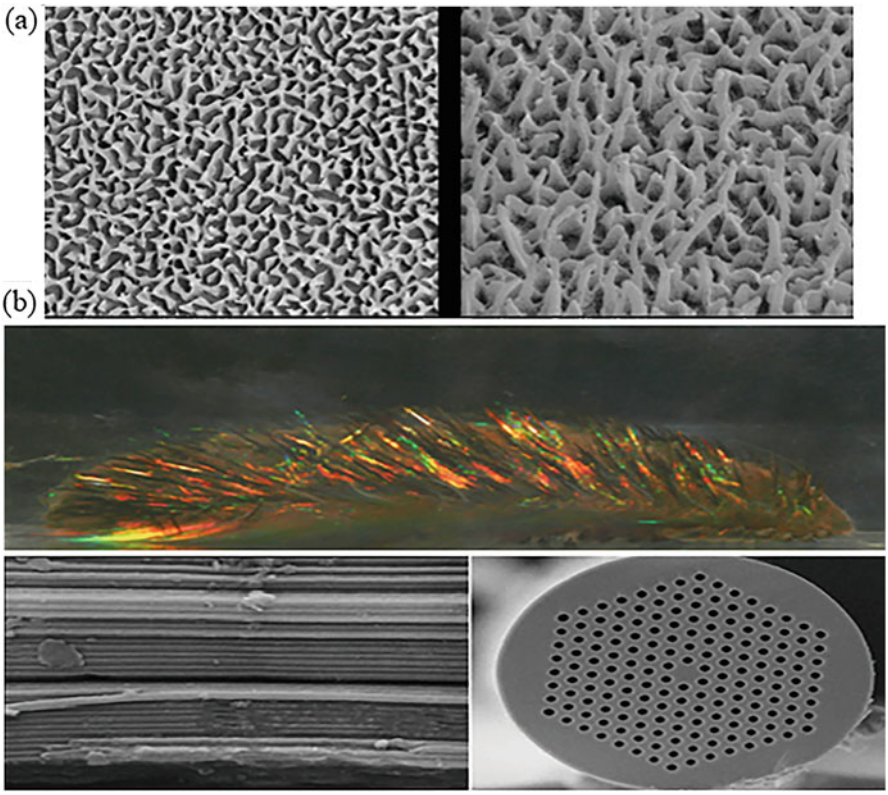

Fig. 3 (**a**) Surface topography of transparent regions of glasswing butterfly. (**b**) The tiny spines of Polychaeta, *Aphroditidae*, are just like inherent photosensitive crystalline materials. The cross-sectional image depicts their closer view, typically comprising regular configured hollow channels with chitin walls (bottom left), the red color of spectrum incident on *Aphrodita* spine. Alternate heating and retrieving the bundles of glass capillaries can be a simpler method to obtain similar synthetic photonic fibers. (Parker et al. 2001; Russell 2003; Siddique et al. 2015; image reproduced with permission)

serves as an incentive for efficient light absorption unlike that of pigmentation. Some notable aspects regarding this property are as detailed next:

- Structural color is a source of tunable energy transformation, and, most importantly, it is environmentally friendly as it does not involve external mediation of chemical dyes. Perhaps, tunable structure exhibition is used by many living organisms as the means of adapting to the surrounding environment such as using communication, predation, camouflage, and several other modulations.
- Several instances can be found supporting the response monitoring via color development. Butterfly wings, hormonal and stimulus responses of plants, flowers, and leaves, environmental adaptation mechanisms of a chameleon, insectivorous plants, and hibernation patterns of certain birds are some other

traits. Butterfly wings comprise multiple beautiful and purely (by themselves) iridescence appearances owing to their hierarchical and ordered repetitive structural constitution. For instance, the butterfly *Morpho sulkowskyi* bears a tempting dual color (blue) on the top and (camouflaged brown) beneath the wing's surface. The distinctive blue and brown appearances are noticed in the flying and landing stages; such characteristics if replicated using pressure equilibration modulations can be the remarkable basis of a piezoelectric biosensor. Apart from being fascinating, the shiny butterfly colors are unique because of the seeing-through (transparent character) ability of their wings. Glasswing butterflies have a characteristic of this ability where the glassy sections of wings confuse their predators, the time lapse in which butterflies fly away. This ability is often recognized as optical transparency, implying that all light that goes into the wing continues out of the other side. A closer look at the transparent regions of glasswing butterflies using a scanning electron microscope (SEM) shows numerous randomly sized patterns of nanopillars (Fig. 3). The exclusive source of the transparent character of butterfly wings (irrespective of directional view) is the random size and shape of pillars. Among the noted erstwhile functions of butterfly wings are their extraordinary hydrophobic and self-cleaning abilities. The significance of hydrophobicity for butterfly wings stems from the inherent ability to resist a rainstorm in the absence of which the residual water on butterfly wings makes flying difficult. The water droplets are unable to penetrate the hydrophobic pockets due to stronger interactions of water molecules than those between water and air. Furthermore, the air pockets have an internal pressure that resists water from permeating through.

- Similarly, *Aphrodite* (a kind of trivial humid water worm) hair possesses intense structural colors derived from front cylinders, which collectively diffract light due to substantial longitudinally oriented, closely packed, and 230 nm in diameter. These hairy structures make up tubular structures constituted of hexagonally configured hollow cylindrical channels that are a few nanometers across and are made up of chitin. These arrays act as 2D photonic crystals that reflect light strongly in the long wavelength region of the spectrum. These structures confer the *Aphrodite* spine, a deep, iridescent red color. Whether the optical properties of polychaete spines have a biological function remains poorly understood, but the applications for light-manipulating fibers in optical technology are indeed immense. Such observations could be traced to the efforts of Phillip Russell

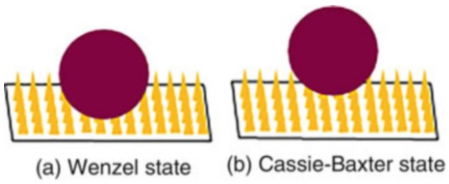

Fig. 4 Surface topology and morphology-driven hydrophobic sensitivity. Due to the larger contact area in the Wenzel state, the role of the surface is more prominent in the functional activities

and collaborators, who fabricate the assemblies by stacking glass capillaries into hexagonally packed bundles and drawing them out under heat into narrow fibers laced across with perforations. If "defects" are introduced into an array of tubular channels (either via including a wider capillary or a solid rod in the bundle), light can pass along the defect while being excluded from the photonic crystals. This creates an optical fiber cladding that is essentially impermeable to the light wavelengths within the band gap. Photonic crystal fibers of such configurations can guide light around the tighter bands than normally possible with conventional fibers, having comparatively weakly confined light due to internal reflection at the fiber surface. Hence, these fibers are anticipated to function better for guiding light in tightly confined spaces, such as on optical microchips.

- Similar examples of such biological structures are the wings of peacocks and the varied appearances of *Hercules* and *Tortoise* (beetles). Monitoring the track record for color-specific tolerated stimulus can be a crucial breakthrough in the utilization of these natural biological mechanisms for optical, electronic, and calorimetric sensing.

2.5 Hydrophobic Receptivity

Hydrophobicity is the most important natural characteristic of natural biological agents (in particular, for the aquatic regime) and endows a sharp dependence on surface topology and morphology (Fig. 4).

Superhydrophobic materials are in typically high demand owing to their potential significance in printing, microfluidic devices, nanoparticle assembly, batteries, high-sensitivity sensors, and optical devices. The following points illustrate the significance of superhydrophobicity in improving the sensing mechanism:

- Feasible strategies toward a progressive replacement of traditional synthetic materials are of key significance. Several natural biological materials are known to possess superwettability. The most fabulous example of natural superhydrophobic biological material is lotus leaves exhibiting superhydrophobicity toward the water with nearly 150° water contact angles and self-cleaning properties. These self-cleaning attributes are immensely useful for the removal of dust and dirt particles through the motional activities of water droplets. Apart from self-cleaning properties, Mele and Feng groups independently reported *Strelitzia reginae* and *Oryza sativa* leaf surfaces to be composed of parallel microgrooves, and also exhibit superhydrophobic features (Wang et al. 2017).
- The anisotropy of superhydrophobic properties enables the displacement of droplets in a direction parallel to that of microgrooves. This response could be a much-needed boost for the development of amicable fluid-transport systems, enabling the assessment of relative aqueous interactions of distinct chemical surroundings of a surface region(s).
- Other fine examples of superhydrophobic biological material are the wings of the *Morpho* butterfly, which exhibit directional adhesion. The nonwetting ability of

these structures facilitates easier shedding of water droplets from a butterfly's wings upon a slight shake, thereby facilitating rain flying. These wings can be easily used to quantify the drying extent of a biological specimen, which is often the requirement for sophisticated characterization sample preparation.

- The genesis of hydrophobicity is manifested through the existence of an air layer trapped inside the rough surface, which can reduce the liquid and ion penetration alongside facilitating efficient heat transfer. Studies have illustrated the making of pollen comprising electronic skin as a hydrophobic biological material with a contact angle of nearly 100°, subsequently elucidating a strong influence of hydrophobic on the electronic signal stability. Investigations focusing (0–30) min water penetration of pollen material also revealed no significant variation in water droplet physicochemical and flow behavior concerning time.
- Modulating the interlayer frictional forces and interlocked structures of e-skin materials through increasing the relative pollen content enabled enhancement in elasticity (a highly important requirement for flexible electronic agent). Thereby, this response illustrated that biological materials are bestowed with several attributes as active units that require consistently good mechanical and hydrophobic properties.

2.5.1 High Adhesion

Smart adhesive materials with flexible surfaces capable of being engineered for various applications are being provided by biological entities. To name a few, biomolecules of amphibian and aquatic organisms (waterproofing ability), the skin of animals, and certain plants are the tissues that meet robust needs. The following points describe such features of several living tissues:

- Owing to strong adhesion or cohesion abilities, biological materials of natural origin are not easy to be de-eliminated from integrated assemblies and are therefore widely used in making multicomponent assembled stable devices. Dauskardt and colleagues provided extensive illustrations of organic semiconductors and devices in terms of their adhesive and cohesive energies, noting the dependence of the former on chemical bonding, van der Waals interaction, and chain entanglements. It must be emphasized here that adhesion may also refer to the contact between two dissimilar molecules/surfaces, whereas cohesion essentially involves interaction between two similar species (which could be the subcomponents of two independent systems) (refer Wang et al. 2017 for cited studies).
- Polydopamine (PDA), herein, is the most famous natural adhesive, depending on both noncovalent and covalent bonding. This material strongly adheres to organic surfaces through covalent coupling under alkaline conditions via appropriate addition or coupling reactions. The process depends on the typical oxidation of catechol (present in polydopamine) to quinines. Steadfast PDA addition has created significant interest in the fabrication of hybrid materials and functional substrates. The past 10 years have witnessed overwhelming importance being conferred to gecko lizards to adhere to surfaces with different orientations and

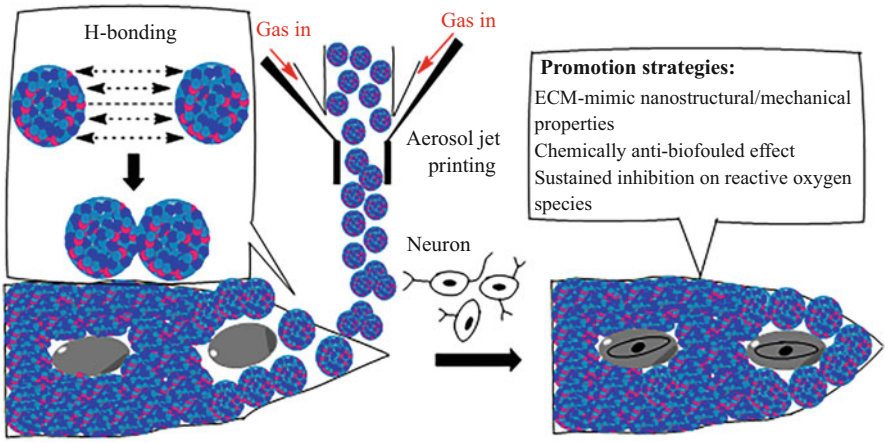

Fig. 5 Schematic procedure of an oligomeric proanthocyanidin (OPC) neural probe fabrication having an anti-inflammatory interface. A native semihydrophilic receptivity of OPC aids in the self-adhesion of nanohydrogels by acting as a structural stabilizer. The aromatic architecture and abundant –OH groups are the key controls for developing a biologically compatible 3D anti-inflammatory neural interface

frictional coefficients. Being a neurotransmitter, such attributes of PDA have revolutionized its use toward efficient binding to its signaling mediators. The relative frictional extents could be therefore utilized in ascertaining the nature of the bound stimulus, keeping the database as standard.

- A 2011 study by Helbig and colleagues revealed the ability of *T. bielanensis* to detect *Candida albicans*, *Staphylococcus aureus*, and *Escherichia coli*, comprising significant diversity in fungi, and Gram-negative and Gram-positive bacteria. Analysis showed no visible culture deposition even after 4 days. In general, terpenes are known to confer protection from plants and insects to safeguard the cuticular surface from microbial adhesion (refer Wang et al. 2017 for cited studies).
- Separate investigations showed that the lipid layer is not covalently connected with the epicuticle and could be therefore extracted using carefully chosen dissolving solvents or mechanical shedding in the form of dust particles through animal movement. Lipids gradually transit to the surface and recover the coating. In this process, the lipid film could be used as a sacrificial layer that is regularly reproduced to avid microbial invasion. So, there is no need to provide nonspecific reaction control from an external agency (to keep microbial contamination arrested) and the growing *T. bielanensis* in itself is capable of protecting against such interference.
- Natural oligomeric proanthocyanidin (OPC), a standard anti-inflammatory drug, can combat neurodegenerative disorders through multiple therapeutic mechanisms. The semihydrophilic OPC assists the self-adhesion of nanohydrogels via acting as a structural stabilizer through its structural architecture and abundant –

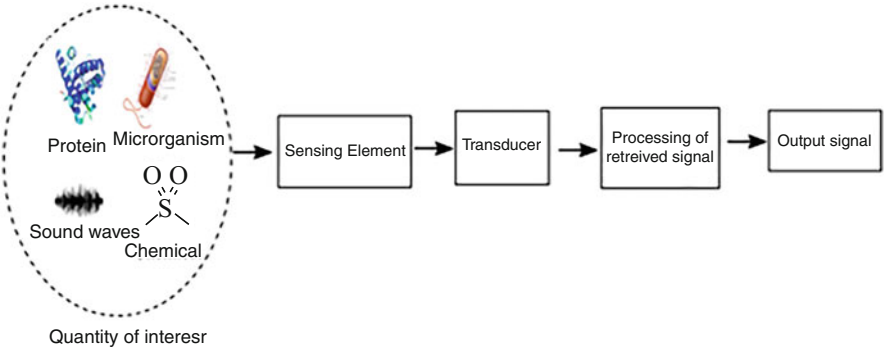

Fig. 6 Flow diagram of sensor functioning

OH groups, which gradually develop into a biologically stable 3D anti-inflammatory neural interface. So, the adhesion ability of OPC provides a strategy for the nanofiber-like protective ability of the hydrogel network via distributing its structural layers and extending the anti-inflammatory impact on the assorted neurons and their exaggerated confirmations.

- Recent studies of the Chen group elucidate a novel 3D nanocarrier utilizing natural antioxidant OPC reagents derived from natural grape (Fig. 5). Consequently, this nanocarrier possesses exceptional antifouling properties as an OPC-coated neural probe, developing lower impedance alongside exceptionally higher signal stability compared to a nanocoated probe after short-range and continual in vivo implantation.
- Inspection of this implanted assembly through immunostaining revealed a reduced astrocyte population around the implanted site through a protective functioning of an OPC-based probe. Apart from decreased astrocyte population, significant reductions were noticed in activated microglia, paving the way for improved survival 28 days after being implanted. Thus, this study presents another remarkable advance through the potentiated antioxidant activity of implanted OPC reagents, which is not only a biological source but also biological essence (as noted through its implantable ability).

3 Sensor and Biosensor Distinctions

3.1 Sensors

A sensor is a device used to detect any variations in physical, chemical, and biological extents quantified through changes in light intensity, force expression or exertion, pressure, locus-defining coordinates, sonication impacts, gases, specificity, and functional activities of proteins, microorganisms, cells, and others (Fig. 6). Enhanced precision of sensing mechanism depends on specificity, sensitivity,

accuracy, cost-effectiveness, and capability to respond in different environmental conditions. While the sensor monitors the interaction of any kind of stimulus (resulting in varied physical, chemical, optical, electronic, or acoustic responses), a biosensor exclusively tracks the changes in the biological response of the scrutinized entity. Based on the physical positioning of the sensing probe and analyte, the following categories of sensors are known to prevail:

- **Contact**: The sensors of this mode necessitate physical contact with probe moiety to sense them. Coziness and biocompatibility are prominent considerations to be

Table 1 Functional attributes of a sensor having a key role in the typical performance

Sensitivity	Stimulus range (span)
Stability (short and long term)	Resolution
Accuracy	Selectivity
Speed of response	Environmental conditions
Overload attributes	Linearity
Hysteresis	Dead band
Operating life	Output format
Cost, size, and weight	Input control

Table 2 The diversity of input stimulus describes a sensor's functioning

Acoustic	Wave amplitude, phase, polarization, spectrum, and wave velocity
Biological	Microbes, enzymatic activity. antigen–antibody interaction, and biomass driven
Chemical	Specific constitutional state of stimulus
Electric	Charge, current, potential difference (mV), electrostatic potential, electric field (amplitude, phase, polarization), and electrical permittivity
Magnetic	Magnetic field strength (amplitude, phase, polarization), magnetic flux, susceptibility, and permeability
Optical	Wave amplitude, phase, polarization, spectrum, wave velocity, refractive index, emissivity, reflectivity, and absorption
Mechanical	Position, acceleration, force, stress, strain, shear, mass, density, torque, shape, roughness, orientation, stiffness, and viscosity
Radiation	Energy, intensity, penetration ability
Thermal	Temperature, flux, specific heat, and thermal conductivity

Table 3 Sensor configuration based on sensed and percept energy stimulus

Sensor configuration	Perceived energy stimulus
Physical	Thermoelectric, electroelastic, photoelectric, thermomagnetic, photomagnetic, thermooptic, magnetoelectric, photoelastic, electromagnetic, thermoelastic, and combinations
Chemical	Chemical conversion, physical conversion, electrochemical process, spectroscopic modulation, and combinatorial phenomenon
Biological	Biochemical conversion, physical conversion, the influence of test organism, and combinations

Table 4 Sensor classification is based on specific applications

Civil engineering	Domestic and structural applications
Distribution, commerce, and finance	Environment, meteorology, and security
Energy and power	Information and telecommunication
Health and medicine	Marine
Manufacturing	Recreation and toys
Military	Space
Scientific measurement	Robotics, bioelectronic, risk perception, safety assessment, and several others

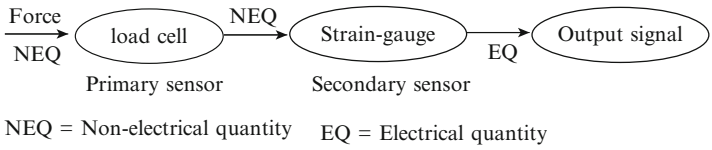

Fig. 7 Distinction of primary and secondary sensors

kept in mind concerning sensor functioning in contact mode, wherein time-bound responses monitor the characteristic performing aspects (of the analyte). A persistent aspect of concern here is the minimization of fouling effects that persist for prolonged durations. *Measuring thermometer.* The most common examples of such sensors include the waterheating electrical rod and temperature. Both of these mandate their residence with their respective sensed moieties (water and body of the host, respectively). Some diagnostic procedures working through noncontact mode include electrocardiography (ECG), electromyography (EMG), and electroencephalography (EEG).

- **Noncontact**: This mode of sensing does not necessitate any direct contact between analyzing probe and the analyte. The most common example of such sensing devices is magnetic retrievers (which attract iron or magnetically sensitive material without physical contact with them). Biological instances of such sensors include the identification of a substrate (among many) by enzyme and antigen (among many) by its specific antibody. These sensors are mostly utilized in ambient applications like passive infrared (PIR) spectroscopy, having less impact on the environment or quantity of interest. A comparative advantage of these sensors over the contact mode sensing devices is that there are much fewer chances (as well as maintenance of provisions) mandating moderation of interaction between probe and analyte, resulting in a better reliability of sensed response.
- **Sample elimination**: This is only a specified domain of noncontact sensors, wherein the prediction of toxins or a threshold extent of a biochemical aspect could be maintained. The purpose of such tracking is not limited to detection but also to entirely squeeze out the undesired proportions. Ascertaining *E. coli*

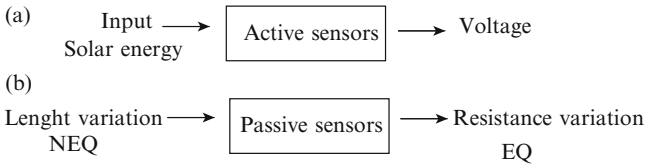

Fig. 8 (**a**) Working diagram of the active sensor. (**b**) Working of a passive sensor

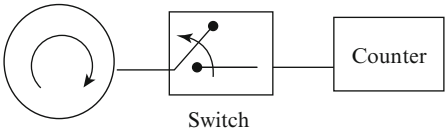

Fig. 9 The switching actions are counted through an electronic counter

contamination of water or excessive blood glucose extents are some illustrations of such sensing mode.

3.1.1 Working Components of a Sensor

- Certain working parameters of a sensor form the defining aspects of its classification, such as sensitivity, stimulus range, constitutional material, detection means, conversion phenomena, the field of applications, and stimulus being intercepted (Tables 1, 2, 3, and 4).
- Sensor performance is a combination of optimized fitting of multiple factors, which are more authentically understood as the classification criterion.

3.1.2 Classification of Sensors

The sensor classification is described as follows:

Classification based on the application method
Classification based on the energy conversion
Classification based on the output signal form

Here is a brief discussion about them:

Classification Based on the Application Method

- **Primary sensor**: Here, the input is directly detected by the sensor. For example, load cell is used to extend the weight of the object.
- **Secondary sensor**: In this case, the output of the primary sensor is sensed by another sensor whereby the nonelectrical stimulus is converted into an electrical signal (Fig. 7).

Classification Based on the Energy Conversion

- **Active or self-generating sensors**: Generates output signal in response to external excitation (Fig. 8a). Examples are LVDT and a photovoltaic cell.
- **Passive sensor**: This produces an output signal without any external excitation. Examples are thermistor and strain gauge produce resistance to temperature and length variations (Fig. 8b).

Classification Based on the Output Signal Form

- **Analog sensors**: Provides varied continuous output signal *vis-à-vis* input changes. In addition, within the sensor's range, the output signal can have infinite values.
- **Digital sensors**: In contrast to analog, digital sensors have a discrete form of output with finite values. Here, the data are digitally converted and transmitted through an electronic counter. For example, the switching actions of a revolution counter (Fig. 9).

3.1.3 Terminological Distinction of Sensors

Though all sensors are exclusively confined within contact and noncontact regimes, some terminologies do refer to them in consideration of their specific property ascertaining. For instance, the optical sensor may indirectly refer to any of the spectrometric techniques. Similarly, ultrasonication subjection could be the means to know about the extent of cavitation meted out for a fixed duration of sound waves persistence in a medium. Figure 10 depicts an approximate distribution of different

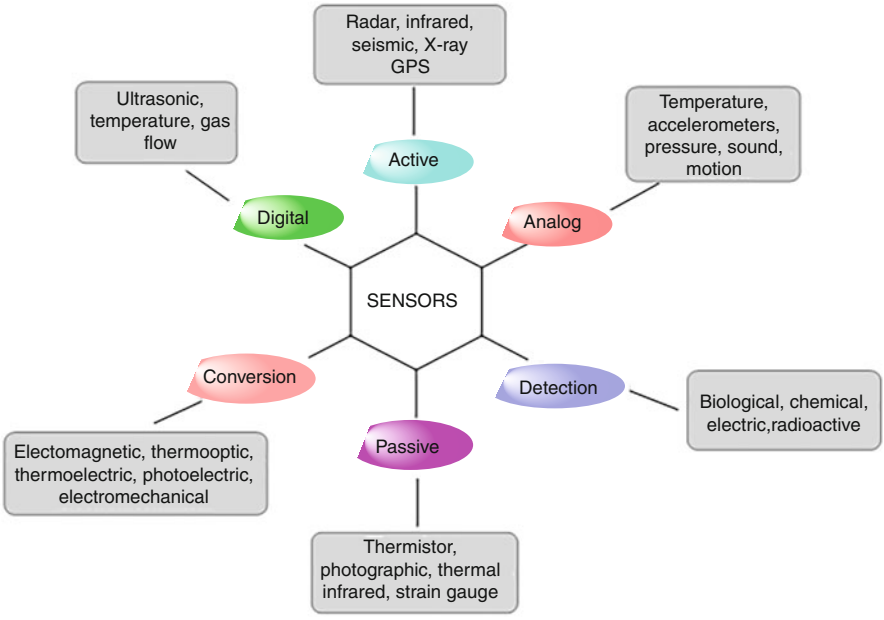

Fig. 10 Brief outline of the sensor types

Table 5 Characteristic sensor types with advantages and disadvantages

Sensor types	Advantages	Disadvantages
Optical	Low cost, small size, and ability to operate over large distances	Short-lived stability
Temperature	Accuracy, flexibility, higher reliability, and sensibility	Resistance error, vibration, and a high response time
Magnetic	High sensitivity, low noise, high switching speed, and reduced power consumption	Shock performance, magnetic metals disturbance, and magnetic field affect the trip point
Image	Reduced energy consumption, security, and digital lock	Slow speed and vulnerability to heating and distortion
Motion	Available at low cost, higher security, and save energy	Sensitive to environment changes, short distance coverage and undesired triggering of motion sensors can occur when it is installed near a light source
Pressure	Easy to rearrange, no parallax inaccuracy, steady readings even at a high vibration, and does not need any operators	The error occurs due to oscillating values, requires a power supply, and is not easy to observe full-scale and trend
Proximity	Well established, easy to operate, high switching rate, insensitive to environmental conditions, and accuracy	Limited operating range, noisy interface, and suffering from the object surface
Ultrasonic	High penetrating power, low cost, high frequency, and sensitivity	It can be affected by environmental changes or conditions
Radar	Insensitive to environmental conditions, direct measurement of vehicle speed, accurate, and reliable	Expensive, difficulty in discriminating between the close objects and color of the objects
Infrared	Effective in detecting defects, and accurate measurement of vehicle speed and position	Incapable of detecting multiple objects having minimum temperature difference and expensive

properties analyzed by the above sensor classification domains through their working modes, energy conversion efficacy, and output signal form. Salient sensor types with their advantages and disadvantages are listed in Table 5.

3.1.4 Sensor Characteristics

- Typically, a sensor response and functioning are described by its characteristic behavior corresponding to static and transient responses toward an input stimulus. These include static and dynamic characteristics.
- The **static characteristics** defining a sensor functioning encompass all properties of a system post-steady-state manifestation of all transient effects, such as accuracy, discrimination, precision, errors, drift, sensitivity, linearity, and hysteresis. The dynamic attributes describe the immediate or instantaneous response of a system to the input. A system can be, therefore, zero, first, or second order in functioning. The following points provide a basic idea of different static and dynamic sensor parameters.

- **Dynamic characteristics**: The response of a sensor to a particular variable input is quite distinct from the one exhibited when the input signals are constant. Dynamic characteristics of a sensor arise due to the presence of energy-storing elements, that is, inertial (masses, inductances) or capacitances (electric or thermal). These parameters are determined by ascertaining the sensor response toward a family of varying input waveforms, such as impulsive, step-based, sinusoidal, or white noise comprising.

A brief explanation of salient static and dynamic sensor characteristics is as follows:

- **Accuracy**: This property of a sensor ensures the retrieval of an output close to that of the true value of the measured quantity. The determination of accuracy is related to the deviation of repetitive measurements and involves absolute and relative errors (Eqs. 2 and 3).

$$\text{Absolute error} = \text{Result-True value} \tag{2}$$

$$\text{Relative error} = \frac{\text{Absolute error}}{\text{True value}} \tag{3}$$

- **Resolution**: This is defined as the least variation in the input needed to effect a detectable output change. In case the initial limit is "zero," the resolution is termed a "threshold."
- **Precision**: This is defined as the progressive ability to repeatedly provide unchanged results instead of a similar input under the definite prescribed conditions. This parameter indicates agreement and coherence between the successive measurements and nowhere means the nearness to the true value. Hence, despite being a necessity for accuracy, it is never a sufficient characteristic.
- Repeatability and reproducibility are the two parameters closely based on precision. The repeatability implies a precision of a set of measurements taken within a short time interval while reproducibility infers the precision of a measurement set taken over a long time interval or the results of experiments done by distinct operators using dissimilar instruments or in distinctive working environments.
- **Errors**: Error could be defined as the deviation from the expected ideal working state for which manifold factors could be responsible. Two major kinds of errors in experimental measurements are systematic and random.
- The systematic errors result from a variety of factors such as modifying variables (i.e., temperature), variations in chemical structure that manifest interactions with the probe, human error (amidst sample loading), and transmission-attributed signal attenuation. These errors could be corrected with the compensation methods (i.e., feedback or filtering).
- Random errors arise due to a signal that is devoid of any information (noise). Factors contributing to the generation of noise include repeatability of experimentalist (could be aggravated by the abnormal height of a rough surface), environmental noise, and transmission noise (typically in the order of 60 Hz).

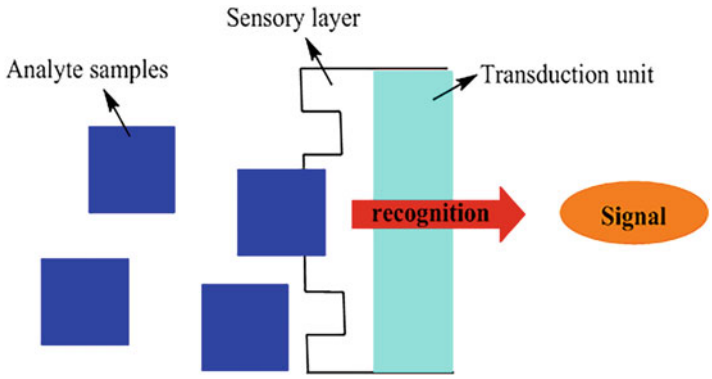

Fig. 11 Schematic representation of biosensor components, wherein analyte samples bind to the probe. The mechanism of sensing is, no doubt, similar to the one depicted in Fig. 6, wherein the recognition element is immobilized using membrane entrapment, covalent or noncovalent binding, which maintains its bioactivity. Apart from this, an indirect process can also be used where a biochemical reaction arises between the analyte and the biomaterial element that results in product formation via the evolvement of heat, gas, ions, and electrons, which are subsequently measured by the biorecognition element (sensory layer) to generate a specific chemical stimulus. The transducers convert this chemical signal into an analogous electrical signal, which is further amplified by the detector circuit

- For a minimized generation of random errors, the signal-to-noise ratio (SNR) should be $\gg 1$.
- **Sensitivity**: This is ascertained as the slope of the calibration curve (discussed in the subsequent section) and should be ideally large and constant. Mathematically, the sensitivity of a sensor is calculated by taking the first derivative of the calibration curve equation.
- **Linearity**: The resemblance extent of the calibration curve to a specified straight line.
- **Hysteresis**: The difference between two outputs corresponding to the same input, depending on the trajectory followed by the sensor.

3.1.5 Sensor Calibration

- The output of a sensor analysis (in response to one or more input stimuli) is usually obtained as a transfer function. Calibration implies checking, adjusting, or determining via comparison with a standard. This infers a comparison between multiple measurements.
- Typically, a sensor calibration involves the application of a range of known physical inputs and subsequent recording of a system's response. The calibration provides desired justification for proper functioning by recording the outputs to manifold input values other than the inputs used in course of calibration. Some potential methods of sensor calibration are as follows:
- Estimation to obtain well-fitted points with the chosen calibration coordinates (curve fitting via computational assessments of nearest trends followed)

- Modification of the sensor properties to fit with determined transfer function
- Optimization of the acquired data by fitting them to a normalized distribution function
- Design of a sensing-specific reference provision having resemblance with select calibration stages

3.1.6 Biosensors

A biosensor is a specific sensor that utilizes biologically derived materials such as an antibody, enzymes, protein, nucleic acid, living cells, and tissues as the recognition element in the transduction process to measure certain chemical concentrations in a biological system (Fig. 11).

3.2 Developmental Background of a Biosensor

- The first-ever use of biosensor terminology was made in 1906 when M. Cremer demonstrated that the acidic load of a liquid varies directly with the electrical potential difference between the separated zones partitioned by a glass membrane.
- Subsequently, in 1999, the concept of pH (hydrogen ion concentration) was unveiled by Soren Peder Lauritz Sorensen and an electrode for pH measurement was designed in 1922 by W.S. Hughes (1922). Between 1909 and 1922, Griffin and Nelson were the first to demonstrate the immobilization of invertase on aluminum hydroxide and charcoal (Griffin and Nelson 1916).
- The first major attempt in the direction of a true biosensor development was put in 1956 by Leland C. Clark Jr., who explained the detection of oxygen. In course of his experiments, investigator Clarke fabricated an "oxygen electrode" that is still recognized as the "Clark electrode" and fetched the regard of "Father of Biosensors Development" Sir Clark (Heineman et al. 2006). This paved the way for

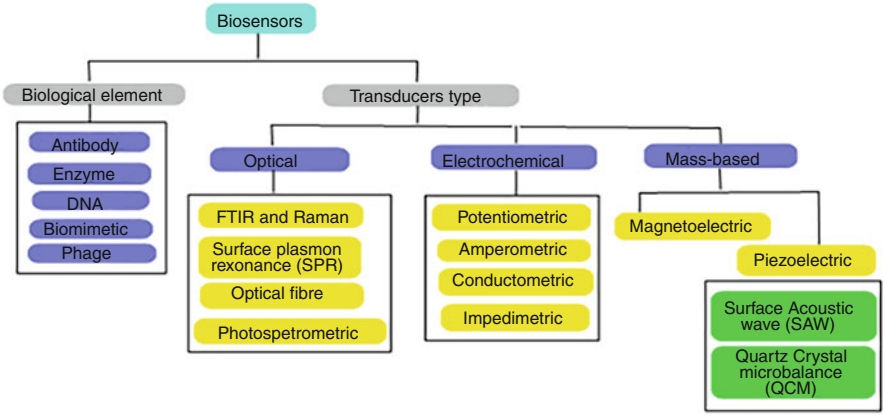

Fig. 12 Biosensors classification criterion

the making of amperometric electrodes working on an enzymatic titer basis to quantify glucose in 1962. Eventually, the first-ever potentiometric biosensor was designed by Guilbault and Montaly Jr. in 1969 for the detection of urea (Guilbault and Montalvo Jr. 1969).

- With gradual developments, the first commercial biosensor was made public in 1975, developed by Yellow Spring Instruments (YSI). Subsequently, with the advances in miniaturized sensing precision, the handheld human blood biosensor was designed by i-STAT in 1992, indicating an era of remarkable progress for biosensor development.
- The subject domain of "biosensors" remains a formidable multidisciplinary research area, involving experts from all branches of basic sciences (physics, chemistry, and biology) and their intensified merger with nanotechnologists, microbiologists, and pharmacists. The decade from 2005 to 2015 lists >80,000 studies on biosensor fabrication and their diversified application domains.

3.3 Classification of Biosensors

- Biosensors are classified into two broad bases, either nature of biological element or transducer type, briefly discussed in Fig. 12. The transducer-driven classification is more rigorous of the conventions as it has an exclusive say to the working efficiency of the biological sensing. Fundamentally, a transducer converts the intervening modifications prevailing in course of interaction between the biological probe and analyte into a measurable signal such as optical or electrical current. The most common transducers for biosensors are electrochemical, optical, piezoelectric, and thermometric.
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3.4 Biosensors Variations Through the Distinctive Transduction Mechanisms

3.4.1 Electrochemical Transducers

- **Potentiometric biosensors** measure potential differences between two reference electrodes separated by a semipermeable membrane. These biosensors are based on an ion-selective electrode and ion-sensitive field-effect transistors (FETs). For example, glucose oxidase can be immobilized on the surface of a pH electrode.
- **Amperometric biosensors** are used to determine the electric current developed via biochemical redox reaction. The working principle of these biosensors involves either the generation or consumption of an electroactive species as the oxidation of glucose in presence of glucose oxidase forms gluconolactone.

Scrutiny of the sensor using a Clark oxygen electrode is used to monitor the depletion of oxygen that is countercompensated by glucose oxidation.

- **Conductometric biosensors** function at comparatively lower amplitude, changing potential difference, and do not mandate the inclusion of reference electrodes. These biosensors eliminate the Faraday principle on electrodes, which generally remains unaffected by light. The best explanation of this biosensing principle is provided by the initial biosensors designed to monitor the kinetics of enzymatic hydrolysis of urea via assessing urease actions in 1965. The functional configuration of this biosensor is comprised of two pairs of the platinum plate, each placed in its explicit measuring cell (with and without enzyme, each). The distinction in the signal response from both cells was monitored through which the possibility of random errors (arising from temperature, buffer, and concentration changes) was reduced. The screening range of urea concentration was within (1–75) mM while that for urease activity was within (0.04–2.5) units per ml.
- Impedance-based biosensors (the principle also being referred to as impedimetric) are made via immobilizing a biological recognition moiety onto a conductive and biochemical electrode. The analyte presence in this configuration was ascertained via monitoring variations in the interfacial impedance. Using biological probes for these sensors involves the administration of a small-amplitude AC potential difference to the sensor electrode. This is followed by measurement of in/out of the phase current response as a function of frequency. The analyte molecules could vary among the antibodies, receptor proteins, single-stranded DNA, aptamers, and peptides.

3.4.2 Optical Transducers

- Based on fluorescence, absorption, internal reflection, surface plasmon resonance (SPR), luminescence, and light scattering intensity.
- In these biosensors, a bound biological moiety on optical fiber interacts with its target analyte and forms a complex having different optical behavior. For example, an immunosensor with SPR activity could be used to detect casein in milk.

3.4.3 Mass-Based Transducers

- In piezoelectric biosensors, crystalline materials such as quartz, gallium nitride, or cadmium sulfide(s) are subjected to elastic deformation or mass variation under the influence of electric potential. This ultimately generates resonant frequency variations in the crystal. Bulk acoustic wave (BAW) and surface acoustic wave (SAW) are two major propagation transducers used in such biosensors. Mechanistically, an acoustic wave is converted into a mechanical wave under the influence of an electric field that propagates either through the surface (SAW) or substrate (BAW). For instance, immobilized monoclonal antibodies (MAbs) interact with their complimentary antigen, resulting in mass density variation as a function of changing the resonant frequency.

- Thermometric and calorimetric biosensors measure the heat generated in enzyme-catalyzed reactions and are exothermic. Measuring heat capacity can be used to determine analyte concentration and also rate of reaction.

Biosensors Classification Is Based on Distinct Biological Elements

Based on the nature of biological entities, biosensors are subcategorized as enzyme sensors, DNA sensors, protein sensors, microbial sensors, and others. Above 80% of the biosensors are electrochemical in nature. Here, biological entities act as a recognition element, having the ability to recognize a single substrate among a group (with different masses).

- **Enzymatic Biosensors**

In these biosensors, the substrate or product of an enzymatic reaction is electrochemically active and capable of being reversibly oxidized or reduced on an electrode upon the application of a suitable potential. They are of two types, namely, substrate and inhibitor biosensors. The former is used to determine specific reaction substrates while the latter is used to screen the agents reducing an enzyme's activity. For example, glucose oxidase biosensor acts as substrate biosensor that determines the glucose, whereas organophosphorus pesticide determination acts as an inhibitor biosensor inhibiting acetylcholinesterase activity.

- **Immunosensors**

Immunoglobulins or antibodies are the proteins generated in response to foreign particles (antigen) by an organism. These antibodies act as receptors and form a strong complex on binding with antigens. Such biosensors are used to detect antibodies having high specificity and selectivity. For instance, the existence of immunoglobulins in the blood sample indicates infection emanated from certain toxic constituents.

- **DNA Biosensors**

Apart from antibodies, aptamers are synthetic nucleic acids that interact with proteins, cells, small molecules, and others. Their high specificity and affinity have made them a promising protein recognition element. Compared with antibodies, aptamers are highly stable and easy to synthesize. These biosensors are used to identify infectious diseases via hybridization between the immobilized oligonucleotide probe and a complementary target sequence, on an electrode. Moreover, DNA sensors are also used to expose several anticancer drugs, tumor markers hampering DNA, and other regulatory proteins.

- **Microbial Biosensors**

In these biosensors, microorganisms are used to detect a target substrate and convert the generated biological response into a physiological, electrical, or biochemical response. Their sensing mechanism depends on conventional optical, electrochemical, and sensory regulated devices. In contrast to the response of enzymes or antibodies, the responses of microorganisms vary as per the culture medium chemical composition. So, in microbial sensors, the biological element is parted from the recording device. These sensors are almost similar to enzymatic sensors, but the only difference is the group of enzymes that take part in converting substrate rather than a single one. These sensors are also known as respiratory biosensors as their respiratory response varies during the assimilation of organic substances. Such sensors are used in determining the concentration of oxidizable organics as well as antimicrobial agents. Other domains of application include toxicological estimation and optimizing the antibiotic dosages. In addition, their enzyme activities and stability can be enhanced using genetically engineered microorganisms. The best example of these sensors is toxin determination through luciferase inhibition, a microbial enzyme catalyzing the substrate with luminescence production. Hence, the different biosensors have significant commercial importance for their wide applications.

To reach everyone's hand, a typical biosensor model should have the following features:

- The biocatalyst should be stable under normal storage conditions and highly specific for the analyses determination.
- Their reaction should be free of physical parameters such as pH, temperature, stirring, and others.
- The response should be accurate, defined, reproducible, and linear over the analytical parameter without dilution or concentration.
- The signal processing unit in the biosensors should be free from any arbitrary electrical noise.
- For clinical studies, these sensors must be biocompatible and sterile.
- Finally, the model should be simple, low cost, portable, and be easily operable by semiskilled operators.

Characteristics of Biosensors

As discussed for sensors (above), certain parameters and properties are mandatory to be optimized for the best biosensor performance. These parameters are therefore recognized as the character traits of a biosensor.

- The working description of biosensor operation is described by its selectivity, reproducibility, stability, sensitivity, and linearity, which are together summed up as the characteristics or "performance-defining" parameters. The following points briefly describe these parameters with the intent to drive an optimum biosensor working efficacy.

- **Selectivity**: Perhaps the most critical characteristic of the typical working mode of a biosensor, selectivity could be defined as the exclusive ability of a biological probe to screen the analyte in varying chemical environments. Thereby, the presence of contaminants of varying chemistry cannot affect the efficiency of detecting a particular molecule. The best illustration of selectivity is provided by antigen–antibody reaction, wherein immobilized Abs on the surface of a transducer are perceived discretely by the antigen comprising solution.
- **Reproducibility**: This characteristic of a biosensor relates to the generation of similar responses corresponding to the detection of an analyte in distinctive chemical environments. The high reproducibility of a biosensor inevitably depends on the precision and accuracy of the transduction phenomenon and electronic sensitivity. Precision implies the ability to generate unaltered outcomes corresponding to multiple attempts of screening an analyte. Contrary to this, accuracy infers a sensor's ability to generate an average output (in multiple attempts) nearing the absolute value instead of multiple measurements. Reproducible outcomes of biosensing are the indicators of high reliability and stimulus adjustment of a typical analysis.
- **Stability**: This property of a biosensor quantifies the susceptibility corresponding to the alterations of distinct sample configurations. These deviations together culminate as differences in the output signals from the standard results (estimated from the database in response to a specific analyte). As a result, estimated titers are accompanied by errors, affecting the precision and accuracy. This property of a biosensor working holds significant relevance for the analysis mandating optimum incubation or monitoring. The operational working of transducers and electronics digitalization must remain unaffected by temperature and other likely involuntary deviations in the vicinity. Thus, appropriate adjustment of electronics is a mandatory requirement to ensure a stable response of the sensor. The affinity of the bioreceptor (the extent to which an analyte binds the bioreceptor) is another factor that could affect biosensor stability. Bioreceptors with high affinities usually result in aggressive electrostatic bonding or covalent linkage of the analyte that compromises biosensor stability. So, it must be ensured that binding activities do not at all induce any structural insult to the native analyte and sensing element configuration. It must also be ensured that the chosen bioreceptor does not undergo any degradation over a due passage of time.
- **Sensitivity**: This refers to the minimum extent of analyte that can be detected by a biosensor probe, also considered the limit of detection (LOD). Several applications from medical sciences to environmental contamination monitoring and others mandate the analyte detection to the extent of nano or even lower ranges per ml of the analyzed fluid. The lower the determined extent, the higher the sensitivity, and ultimately least chances of contamination would remain. For instance, the general recommendation of biopsy tests by doctors in response to prostate cancer is made subject to a minimum 4 $ng \bullet ml^{-1}$ prostate-specific antigen (PSA) blood concentration.
- **Linearity**: This characteristic of a biosensor ascertains the accuracy of a measured response (corresponding to a set of measurements in response to varying

analyte concentrations). Ideally, a graphical dependence of the output signal on the analyte concentration is deemed as a straight line, $y = mx$, where y is the output signal, x is the analyte concentration, and m is the biosensor sensitivity. The linearity of a biosensor is associated with its resolution and the range of analyte concentrations being examined. Resolution of a biosensor could be defined as the smallest change in analyte concentration deemed suitable to effect a response variation. Although it varies with the application concerned in general, a good resolution argues well for biosensor performance as the use of biosensor not only pertains to analyte detection but also determining its concentration over wide working ranges. Extensive studies monitoring a biosensor response define the corresponding linear range, which refers to the range of analyte concentrations corresponding to which a biosensor response varies linearly with concentration.

4 Improving the Working Efficacy of a Biosensors

The typical working efficacy of a biosensor is discretely affected by the optimal contribution of each of its above characteristics. In convention, the electrically active mammalian cells cultured on extracellular electrode arrays are used to detect bioactive agents. As cells are easily affected by varying environmental conditions, environmental and biochemical variations can easily trigger cellular responses that contribute to noise in a mammalian cell-driven biosensor. Thus, it is highly essential to maintain the growth supporting pH, temperature, and osmolarity so that the native detection sensitivity of living cells is not altered and could be rightly exercised. It is observed that the response of a growing mammalian cell is significantly distinct through its different growth stages, owing to growth or instantaneously specific biochemical secretion by a host cell. Other than environmental and biochemical factors, the performance efficacy of a biosensor is also affected by the distinct recognition of two closely resembling stimuli. For example, distinct recognition of microbial stains collected from the air and infected sufferer is significantly cumbersome and the one from the air is easily accepted to be much more heterogeneous. Similarly, the detection of proteins produced by a microbial cell and an agricultural crop plant mandates the right selection of distinctive markers for optimum assay design. Thus, for a distinct recognition, it is a must to identify distinguishable markers in the screened cells. With the advancement in characterization techniques, it has become quite handy to screen the distinctive markers among multiple sources of a similar biostimulus. Apart from discrete markers, efficient and prompt functioning of recognition and transduction domains of a biosensor is highly desired.

The following are some aspects of the modulation of which the rapid and implicit response from a biosensor could be optimized:

1. Enhancing detection sensitivity
2. Incorporation of nanomaterials
3. Reduced detection time and analyte quantity
4. Reusable substrate–analyte interactive platforms

A brief discussion of all these parameters is provided next.

4.1 Enhancing Detection Sensitivity

Detection sensitivity (DS) infers the minimal intensity of the input signal distinctly recognized by a sensing probe. This could also be considered as the least count extent of a designed sensing configuration. In practice, every scientific instrument or probe has a defined workable range, and sometimes for the measurements beyond these extents, accessories in the functional setup are combined. A perfect example of DS-based biosensors differentiation could be the distinctive diagnosis protocols for screening more than one kind of infection. The protocol used for screening jaundice provides a negative response if used for diagnosing dengue or typhoid. The programmable mechanisms of each diagnostic kit are tuned specifically concerning characteristic antigens and are highly optimized for specific responses. Now, if we compare the detection mechanisms specified to a particular response, we notice that the recognition efficacy of two or more probes differs from each other, like the response time of human and mouse brains to a similar external shock or even between two human beings belonging to a different geography. Likewise, the time taken by an electric rod and gas heater to heat two water samples having identical initial temperatures is quite different, owing to the dissimilar DS. So, the distinctive probe configuration concerning screened analyte manifests the presumably distinctive DS. Some primitive aspects of DS are described next.

- The probe–analyte interactions in a biosensor working are strictly physicochemical and therefore highly delicately influenced by probe geometry (size and surface area). However, detection is not merely dependent on physical contact and is perceived only by the generation of a signal in response to the perceived signal. A fine illustration of DS could be gathered by the understanding of the Vernier caliper (VC) and a simple plastic scale. While both are designed to measure length, the former has a lower least count. Similarly, chemical indicators (used for screening acidity or alkalinity) respond variably to a reached or modulated pH level.
- We observe that in both instances the probes provide varying inputs, although they are prepared to perceive a similar quantity. Interestingly, the response time in screening a stimulus is not at all dependent on the analyte configurations but on the programmed working algorithm of the probe.
- While both the discussed instances involve physical entities (electric heater-rod and Vernier caliper-plastic ruler), the mammalian cell-based sensors are quite distinct as the living cells are to be used as probes. The typical response in such cases is the outcome of implicit probe–analyte interactions, and it is obvious to anticipate a faster response for a stronger analyte–probe interaction. So, if an analyte interacts strongly with a probe, it is very much likely that it would be perceived easily. For example, glucose oxidase perceives glucose from the blood and several other mixed solutions, and thereby the DS of a mammalian cell-based

sensor working through glucose oxidase as a probe would be highest for glucose than other carbohydrates or proteins.

- The analyte concentration and texture are crucial parameters affecting the DS of a probe. Although the probe is implicitly specific to an analyte, the latter's high proportion in the scrutinized sample serves as a positive factor for prompt detection. The morphology or texture of the analyte is a highly vital prospect affecting an analyte DS, wherein a nano-thin analyte layer is much easier to be detected rather than its bulk form. This is because the energy levels in a nanomaterial are continuous and the possibilities of intramolecular transitions are much higher.
- Enhanced DS is of critical importance in diagnostic purposes as the distinctive ascertainment of physiological infections is of paramount importance to advise or suggest the cure. So, the right interaction of probe and analyte forms the key to providing a reliable detection response. It is practically very much infeasible to expect that two or more biosensing responses develop similar DS with the same probe. Though, it is possible that certain modifications of the probe could reduce the detection time of analyte recognition. This is attained through nanotechnological interventions, wherein NMs having infinitesimal closer energy levels (owing to a feasibility prevalence of quantum mechanics) could perceive even a slight change in the surrounding environment with a prompt change in their characteristic response. The following section discusses the various NMs being used to improve the sensing probe detection efficacy via prompt recognition and substantially reduced times.

4.2 Incorporation of Nanomaterials

- The realm of materials chemistry accompanied by sophisticated characterization tools forming most fundamental links to explore the possibilities at atomic-scale resolution has been the reason to make the best use of enhanced surface area and reduced activation energy of NMs for accomplishing a faster response generation.
- Manifold NMs such as metal nanoparticles (NPs), carbon NMs (carbon nanotubes, fullerenes, quantum dots), and functionalized assemblies of distinctive materials are being used with increasing interest to fasten the sensing interactions and reduce the response times for a prompt detection mechanism.
- A prominent feature of all NMs is their high specific surface area (surface area to volume ratios), which aids in the attainment of the enhanced extent of bioreceptor units. While using NMs with the biosensors, a consistent challenge has been the immobilization-driven conjugation of a biospecific entity over the NMs. Readers are suggested to refer Putzbach and Ronkainen's 2013 contribution to the different biofunctionalization mechanisms of NMs.
- The major noncovalent approaches to mediate the conjugation of nanomaterial and biological entities are electrostatic interactions, π–π stacking, polymeric entrapment, and van der Waals forces. These binding interfaces exhibit the desired properties of a nanomaterial as well as a biomolecule. While the covalent binding

is often recognized as the regulatory force for biomolecules–nanomaterial attachment, distinguished via stability, reproducibility of surface functionalization besides decreasing nonspecific physisorption. A potential concern with covalent biomolecule–nanomaterial linkage is the unrestrained anchoring that is probable to affect the recognition determining domain.

- Immobilization of biomolecules by supramolecular or coordinative interactions has garnered significant recognition over the past few years for assisting the binding of biological species to surfaces. A familiar instance applied for biosensor engineering comprises biotin/avidin (or streptavidin) system (Wilchek and Bayer 1988). This mechanism works by the attachment of biotinylated molecules to biotinylated surfaces via avidin bridges. Other affinity configurations are also reported such as nitrilotriacetic acid (NTA/Cu^{+2}/histidine complex) or the host–guest system (adamantine/β-cyclodextrin) (Haddour et al. 2005). The preferred benefits of such systems over other immobilization methods include reversibility and the possibility of regenerating the transducer component. The functionalized transducer surface and altered bioreceptor configuration could be separately prioritized on a one-to-one basis, thereby assuring reproducibility.
- Depending on specific chemical compositions, nearly all NMs can be functionalized either via the direct route (during the synthesis) or by coating functional polymers without altering their specific characteristics (Biju 2014). Thus, the functionalization in NMs not only enables a reproducible immobilization of receptors but also improves biocompatibility.
- A major advantage of the use of NMs in biosensing is the progress of label-free transduction mechanisms, together contributing to obvious signal amplifications upon being used as labels. Such ability of NMs resolves the hurdles caused by no direct detection of recognition event by the used transduction technique (the usual observation in course of antigen–antibody immunoreactions or amidst the hybridization of complementary DNA strands). In both these cases, biologically specific constituents (secondary antibodies or DNA strands) modified with optical or electrochemical transduction-compatible labels need to be used.
- Most well-documented and reported NMs used to improve the performance of the biosensor include gold (Au) NPs, quantum dots (QDs), magnetic nanoparticles (MNPs), and the nanostructures of carbon.
- The salient aspects of each of these entities concerning improved biosensor performance are described next.

4.2.1 Gold Nanoparticles

- The major reasons for the increasing application of gold (Au) NPs in biosensor development are their relatively high biocompatibility, size and shape tunable optical and electronic properties, robust preparation methods with significant reproducibility, and, most importantly, the ease of being functionalized with several biomolecules.
- Belonging to the 11th group and the 6th period of the periodic table, Au has a stable electronic configuration with a filled 5d orbital (10 electrons) and a half-filled 6s orbital. Several methods are known for the preparation of Au NPs, of

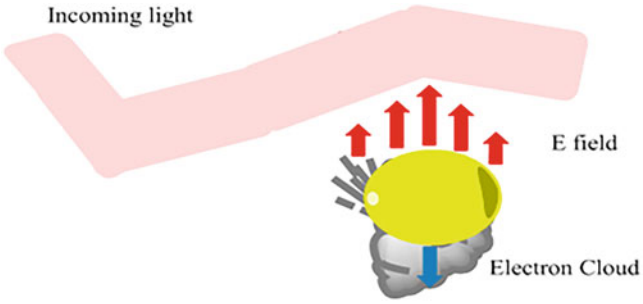


Fig. 13 Pictorial depiction of polarized electron density corresponding to the resonant excitation wavelength. Excitation with wavelengths lower than the particle dimensions prevented the propagation of surface plasmons along the surface of Au NPs, inducing the polarization of electron cloud in a peculiar periphery of the particle

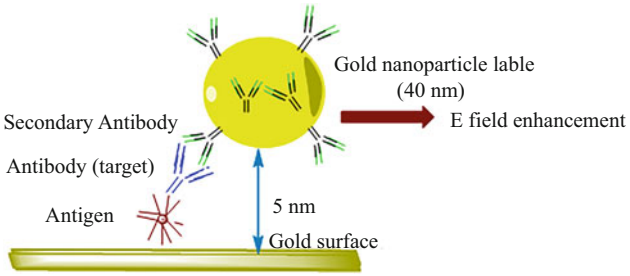


Fig. 14 Representation of attenuated propagating surface plasmons on Au surfaces provoked by defined size Au NPs, which are at a finite distance, leading to a surplus change of evanescent field and ultimately to an enhanced signal

which the most prominent is the approach proposed by Turkevich and coworkers. This method uses $HAuCl_4$ as the Au precursor salt and trinitrate as a reducing agent. The method is a chemical reduction approach, and its several modifications (using distinct reducing agents) are also known. In the mechanism proposed by Turkevich and colleagues, it is well reported that varying the combination stoichiometry of precursor (here, $HAuCl_4$) and the reducing agent could provide variations in the sizes of generated NPs. A lower size generally argues for a greater nanoscale effect (high energy conversion) due to greater manifested quantum confinement. It is due to high native stability that NPs of Au exhibit significant potential to immobilize mammalian cells with minimal risk of cross-reactivity and toxic responses (Turkevich et al. 1951).

- Among the manifold sensing incentives of Au NPs is the optical behavior, wherein irradiation with a specific wavelength of light oscillates the electrons in the conduction band, referred to as resonant surface plasmons. Of note, every material comprises an energy barrier separated by valence and conduction bands. At the ground state, all constituent electrons lie in the valence band but on gaining energy (as temperature, interacting moieties, etc.) these gain entry in the

conduction band. The mechanism by which size-dependent optical attributes of Au NPs aid in obtaining an improved sensing response is optimized by the interaction of incident light wavelength with smaller particle size.

- The interaction of such a smaller NP size with the incident wavelength forbids the propagation of oscillating electrons along the surface (unlike conventional SPR). This results in the electron density polarization on one side of the particle with a simultaneous oscillation of plasmons in resonance with the light frequency (Fig. 13). This experience is sensitively dependent on NP size and shape as well as the dielectric constant of the environment.
- The environmental dependence of such light–matter interaction comprises a substantial gain for bioanalytics as the recognition can result in varied oscillation frequency that ultimately results in a color change of Au NPs, on being observed with the bare eye. Several robust and modestly functioning colorimetric biosensors are reported for DNA or oligonucleotides detection.
- Au NPs are also preferred for bioanalysis through SPR transduction and through a change in the dielectric constant of propagating surface plasmon environment of Au films. This facilitates the detection of analyte(s) in multiple manners such as through changes in the angle, intensity, or reflected light phase.
- A modification in the above technique could be mediated using the Au films and NPs in a sandwich regime. The surface plasmons on Au NPs instigate a disturbance of the native optical field of Au film besides immobilized bioreceptors unit and recognized analyte. Figure 14 pinpoints the optical configuration of this approach for <40 nm Au NPs working at a 5 nm separation from the Au film surface. Here, Au NPs function as labels on being attached to secondary antibodies or DNA strands. Despite further preparative steps being needed for a labelless detection, such an approach facilitates a signal enhancement by several orders of magnitude.
- Au NPs can also aid in the generation of a signal transformation, facilitating discrete single-molecule detection. The working module is activated by a perception of the refractive index of localized surface plasmon resonance (LSPR) conjugated with enzyme-linked immunosorbent assay (ELISA) through 60 nm NPs. Procedurally, horseradish peroxidase (HRP) was immobilized on Au NPs through biotin–streptavidin conjugation. Detection is made using the principle illustrated by Chen and associates, wherein HRP oxidizes the soluble monomer, 3,3′-diaminobenzidine (DAB), to insoluble and colored polybenzimidazole. This reaction resulted in aggregation in the enzyme surroundings and facilitated the precise detection of even a single HRP molecule bound to Au NPs. In this way, Au NPs spectrum and excitation of energy levels are well suited for sensitive biosensing aided using surface-enhanced Raman scattering (SERS).

Based on surface plasmon-enabled signal amplification of adsorbed or immobilized compounds' vibrational spectrum, it is possible to reach the detection range to an extent of a single molecule. Regarding formal restrictions of the reference inclusion, the studies referred to or recalled for the above facts and accomplishments could be traced to the 2014 *Frontiers in Chemistry* contribution of Holzinger and colleagues (Holzinger et al. 2014).

- Apart from interesting optical properties, Au NPs also exhibit an inherent ability to transfer electrons across a wide range of electronically responsive biological species and electrodes. This attribute of Au NPs is in high demand for redox enzyme-assisted biological sensing having the bioreceptors capable of catalyzing the analyte oxidation or reduction. The distinguishing aspect of such Au NPs-assisted redox signaling from that of conventional electrochemical enzyme biosensors is that in the normal setup the generated species are oxidized or reduced to generate an electrochemical signal. A major issue with this methodology is the diffusion of noticeable molecules to the electrode from where a considerable extent is lost in the solution. This hurdle is resolved by the electron shuttle-like behavior of Au NPs, that is, the NPs could approach the redox center of the enzyme, regenerating the biocatalyst by transferring the electrons (in the redox reaction) to the electrode (Fig. 14).
- A significant attempt to improve the sensitivity of the biosensor using 20 nm colloidal Au NPs improved the DS of an amplitude-sensitive paired surface plasma wave biosensor (PSPWB) from 0.001% sucrose in an aqueous state and subsequent biomolecular interaction of 10 pg•ml^{-1} mouse IgG with anti-mouse IgG. It was noticed that 20 nm colloidal Au NPs conjugated with target molecules enabled a greater mass coverage with a larger resonant angle change of plasmon resonance, resulting in a significant enhancement of DS. The uncoated Au NPs, which are randomly suspended in solution, enabled a distinct recognition of biologically specific binding-induced signal enhancement. The Au NP-conjugated protein A (PA-Au) on interacting with mouse IgG (immobilized over a CM5 sensor chip) was screened with a 330 fg•ml^{-1} sensitivity, thereby enabling nearly sixfold signal amplification compared to similar PA concentrations that are not conjugated with Au NPs (Hsieh-Ting Wu et al. 2007).
- The feasibility of multiple functionalization modes enables Au NPs to function well as combination probes facilitated via distinctive interactions. For instance, hybrid electrodes using Au NPs in combination with silicon oxide, carbon nanosphere, and calcium carbonate have been recently reported to enable synergistic responses in analytical performances (Li et al. 2010). A different study by Cai and colleagues demonstrated a ratiometric electrochemical method using polythionine-Au as an electrode. The developed immunosensor exhibited an enhanced specificity over a wide linear range with 2.2 pg•ml^{-1} LOD (Cai et al. 2016).
- Thus, the outstanding tunable and optimized properties of Au NPs propel them as promising candidates not merely in bioanalytics through manifold mechanisms. Such abilities of Au NPs are largely due to the shape and size-dependent properties, allowing an accomplishment of desired applications. The performance attributes represent the manifestations of discrete optical, catalytic, and electronic responses of Au NPs.

4.2.2 Quantum Dots

- Arguably the most exciting NMs, QDs typically constitute the luminescent semiconducting nanocrystals (NCs) having 3D restricted motion of charge

Fig. 15 An illustration of QD sorting in accord with size-emitting light of different colors excited simultaneously by a single-excitation wavelength

carriers. This restriction is termed "quantum confinement (QC)," a typical determinant of nanoscale influence in the material dimensions. The QDs, with 3D QC, exhibit a restricted motion of charge carriers in all the three dimensions. Owing to this, the fascination toward their biosensing application draws interest from their size-dependent optical and electronic energies. This is the reason for different colors of varying QD sizes. Their use in biosensing revolves around the principle of labeled antibodies to which the test antigen binds and results in the binding intensity proportional to color development. The exhibited color is an indication of perceived binding energy from the antigen–antibody interactions. The native electronic behavior of a QD is semiconducting. Readers are suggested to consult the 2009 review article by Drbohlavova and colleagues to know about the referred articles in this section unless specifically stated otherwise (Drbohlavova et al. 2009).

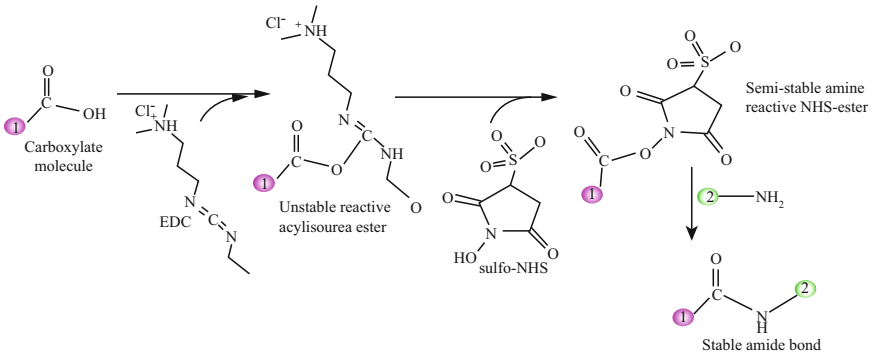
- Two approaches for QDs' preparation are well demonstrated to date. The first involves a form of semiconductor NPs through colloidal chemistry while the second one involves the epitaxial growth using lithography. The colloidal chemistry mechanism involves the rapid injection of semiconductor precursors into hot and vigorously stirred specific organic solvent containing molecules capable of coordinating with the surface of precipitated QD particles. This mechanism is facile and is referred to as a single-step method in many literature sources. For biological systems, QDs are extensively used in the solution phase. Many studies have documented an urgent need for the deposition of QDs on multiple solid surfaces for biomedical applications. In this context, an alternative promising approach for biological applications is to use QDs labeled biofunctional carrier spheres.
- Optical characterization is generally accomplished using UV-Vis and photoluminescence spectroscopy, offering a rapid, safe, and contactless option. The optical attributes are majorly inferred through fluorescence emission and can be fine-tuned by size variation, a prominent factor that ascertains spectral position and photoluminescence purity extent. The size estimations of QDs are usually made through SEM, transmission electron microscopy (TEM), and dynamic light scattering (DLS). One study by Gu and colleagues reported the size and composition of optically active CdZnSe/ZnBeSe QDs through photoluminescence, photoluminescence excitation, and Raman scattering spectroscopies combined with photoluminescence and LO phonon energies model (refer Drbohlavova et al. 2009 for discussed studies).
- In general, the QD size ranges from (2–30) nm though some literature sources list the diameter as strictly less than 10 nm. Typical dimensions exclusively depend

on the used material for their preparation. Evidence and consensus for calling a nanoparticle QD is the prevalence of QC, present when an NP radius is lower than one of the Bohr electron, hole, or exciton radii. Since the Bohr radius varies from one to another material, it would therefore not be right to coin an NP as QD merely based on its size. Constitutionally, QDs can be metallic (Ni, Co, Pt, or Au) or semiconducting. The reduced dimensions of QDs are the manifestations of significantly distinct characteristics compared to bulk solids, arising from the concomitant quantum confinement effects (Fig. 15).

- Thus, by varying the NC sizes, a large range of emission wavelengths could be detected, thereby supporting efficient multiplexed analysis using conventional optical transduction. The concerns in the detection efficacy might be incurred owing to structural defects in the crystal lattice that trap the excited electrons or holes and ultimately result in nonradiative relaxation.

To counter this, the composite morphology is adopted, wherein wider band gap energy material is composited with the native QD and the surface defects are hindered. The high photochemical stability of such QDs' architecture has emerged as a promising alternative and amicably replaced organic fluorophores (Resch-Genger et al. 2008).

- To date, one of the most studied QDs for biosensing purpose is comprised of cadmium chalcogenides (S, Se, and Te), having a significant absorption spectrum and a size-dependent narrow emission spectrum. Such absorption and emission attributes of QDs are the outcomes of their characteristic valence and conduction band gaps, which in turn are the implicit functions of crystal sizes. This energy gap is a quantitative estimate of electronic excitation from the valence to the conduction band with a smaller crystal size corresponding to a high energy gap, resulting in distinct emission wavelengths arising out of electron–hole recombination (Poznyak et al. 2004). The latest progress in QDs' design provides them



Scheme 1 The EDC–NHS coupling reaction for manifesting carboxylic group as stable amide linkage. The method utilizes a hydrophilic probe to manifest its –COOH functional group as $–NH_2$ linkage of the product

with inert or biocompatible coatings, whereby nearly every biomolecule could be attached to these without any undesired effects on photophysical recombination.

- QDs exhibit significant promise in biosensing arising out of their unique physical and optical attributes that facilitate an attachment of multiple biomolecules on their surface. Several assays of QDs are in practice having improved the conventional detection methods of DNA and protein. For instance, the detection of adenosine triphosphate (ATP) using a QD-tagged nucleic acid bound to manifold molecular targets (thrombin, adenosine, or cocaine, to name a few) has been described. Biomolecules can be bound to QDs' surface either directly (via covalent or noncovalent mechanisms) or through an intervening stabilizing layer that acts as a cross-linker between ligand and reactive NP surface. Noncovalent and direct binding can be accomplished through an electrostatically coupled strategy. Compared to the more common covalent mechanisms, the noncovalent self-assembly is simpler and easy to reproduce and accomplish. Using this approach, cysteamine-stabilized CdTe QDs have been reported to bind the single-stranded DNA via electrostatic attraction between positively charged $-NH_2$ groups on QDs' surface and the negatively charged phosphate backbone of DNA.
- The covalent bioconjugation mechanism works via the replacement of thiol acids on QDs' surface with thiolated biomolecules. One study using this binding mechanism has reported covalent linkage of streptavidin maleimide or to conjugated transferrin and mouse anti-human CD71 MAb to CdSe/ZnS QDs.

The use of hydrophilic 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) for forming QD–protein covalent conjugates is a familiar coupling method. The coupling aims toward the use of the hydrophilic compound as a carboxyl activating agent for coupling primary amines to form amide bonds (Scheme 1).

Using the EDC–NHS coupling probe, Wang and the group reported the conjugation of hydrophilic 3-mercaptopropyl acid-stabilized CdTe NPs with the peptides or proteins mediated by NHS.

Fig. 16 Mouse embryonic stem cells labeled with six distinct QDs were subcutaneously injected on the back of a thymic nude mice on immediate injection. (Adapted from Drbohlavova et al. 2009)

- The coupling of biomolecules through QDs is more easily accomplished by the core–shell configuration, typically characterized by a CdSe core enclosure in a ZnS shell. Under these configurations, the core is a typical identifier of the band gap and the consequent emission pattern. Since the red shift in the optical spectrum is well known for high particle size, emission characteristics of a QD are modulated via an appropriate selection of material whose core size can be altered. Likewise, the emission spectra could also be changed through varying the Cd to Zn proportion but not the overall NC size at all. Based on such traits, multiple core–shell configurations of QDs are known, having the core comprising an emissive semiconductor (CdSe, CdTe, etc.), which is capped with a thin shell of higher band energy gap material (ZnS, CdS, ZnSe, etc.).
- There are multiple ways in which one can use QDs for biological cell/tissue labeling and cellular imaging, both in vitro and in vivo. Hybrid nanostructures comprising the QDs–liposomal assemblies are being swiftly developed and are studied for efficient uptake ability by the living cells in case of defective apoptotic controls. Such structural assemblies are best suited as fluorescent probes for ex vivo labeling using hydrophilic QDs with no other changes. In a related study, Kerman and colleagues demonstrated a sandwich immunoassay made up of QD-labeled assemblies for detection of total prostate-specific antigen (TPSA), a prominent tumor marker on a screen-printed carbon substrate.
- Modifications are also well known for the use of QDs in molecular tracking via immunohistochemistry, affably substituting the fluorescent beads used to ascertain the motion of neurotransmitters. The (10–20) nm dimensions of molecular detection-suited QDs contrary to that of 500 nm for the latex beads, allowing an in-depth analysis of an individual receptor's lateral movement. The use of QDs has also been illustrated for the detection of genetic diseases via combined imaging with stage-specific scanning confocal microscopy (SCM), which traces the QDs not exhibiting any chromatin aberration, with a resolution of >10 nm.

Infrared QDs have also been studied as handy probes for noninvasive in vivo detection, largely within small animals, substituting the poorly stable and low quantum yield of conventional IR-sensitive organic fluorophores (Fig. 16). Likewise, Lin and colleagues studied the in vivo multiplex imaging of mouse embryonic stem cells labeled with peptide-based Qtracker (a dye)-delivered QDs and noticed that QD labeling of mouse ES cells did not adversely affect the viability, proliferation, and differentiation.

- QDs have also been used in the nucleus labeling within the live cells. The studies of Chen and Gerion deserve a mention here, wherein it was noticed that nuclear localization signals (called viral peptides) conjugated with CdSe/ZnS QDs did not exhibit any toxic response within the HeLa cells. Similarly, Lieleg and colleagues developed a specific method of labeling membrane integrins in living osteoblasts using functionalized QDs. The investigators used cyclic Arg-Gly-Asp (RGD) tripeptide sequence and a biotin–streptavidin linkage to specifically couple individual QDs with integrin. Interestingly, even though observations revealed a

Table 6 The different configurations of magnetically sensitive materials used for biological sensing

Type of magnetism	Atomic/magnetic behavior	Examples
Diamagnetism	Constituent atoms have no magnetic moment Susceptibility is small and negative(-10^{-6} to -10^{-5})	Inert gases; metals such as Au, Hg, Cu; nonmetallic elements, e.g., B, Si, P, S; many ions such as Na^{+}, Cl^{-}, and their salts; diatomic molecules like H_2, N_2, H_2O, most organic compounds
Paramagnetism	Constituent atoms have randomly oriented magnetic moments Susceptibility is small and positive (10^{-5} to 10^{-3})	Certain metals, e.g., Al, some diatomic gases, e.g., O_2, NO, ions of transition metals and rare earth metals and their salts, rare earth oxides
Ferromagnetism	Atoms have parallel aligned magnetic moments Susceptibility is large (below T_c)	Transition metals Fe, H, Co, Ni, rare earth with (64–69) ranged mass number, alloys of ferromagnetic elements, some Mn alloys, e.g., MnBi, Cu_2MnAl
Antiferromagnetism	Atoms have antiparallel aligned magnetic moments Susceptibility is small and positive (10^{-5} to 10^{-3})	Transition metals like Mn, Cr, and several of their compounds, e.g., MnO, CoO, NiO, MnS, MnSe, Cr_2O_3
Ferrimagnetism	Atoms have mixed parallel and antiparallel aligned magnetic moments, below $T = T_c$, magnetic susceptibility is high	Fe_3O_4 (magnetite), Fe_2O_3 (maghemite), mixed oxides of Fe, and other elements such as Sr

significant decline in the number of blinking QDs but simulations inferred no harm of blinking QDs to quantitative screening of integrin trajectories.

- Erstwhile studies have demonstrated the luminescent cell marker-equivalent functioning of QDs, having an ability to discretely identify the specific molecular structures. The use of multicolor cellular labeling with QDs is already well-established, involving receptor-mediated uptake or via random endocytosis that employs the passive transport strategies to transport NPs across the cellular membranes. Such incentives eliminated the risk-prone external material cellular transfer methods of microcapillary injection and electroporation (using cell membrane mechanical defects to penetrate across cell membranes), in turn creating awareness about the comparatively safer endocytosis approach.
- Thus, the sensing capability of QDs is substantially mediated by optoelectronic and medicinal applications. Swift efforts are in progress to enhance the biosensing applications of QDs by reducing their toxicity. The attempts in this regard include a coating of QDs surface with a protective and stabilizing shell so that they could be safely used for bioconjugation with proteins, peptides, or other chemical moieties. An important observation has been the high stability of the round shape of QDs for their biosensing applications using them in the solution/colloidal phase.

4.2.3 Magnetic Nanoparticles

- Among the most promising alternatives to fluorescent labels in biosensors, MNPs are unique entities exhibiting characteristically distinct magnetic behavior compared to bulk material due to a reduced number of magnetic domains. Magnetic domains refer to regions of parallel magnetic moments caused by the unpaired interacting electrons in an atom. The fundamental source of magnetic force is the movement of electrically charged particles.

So, the magnetic behavior of a material is a direct function of its atomic structure. Electrons in an atom exhibit two kinds of motions, one a planetary motion around the nucleus while another is a motion about their spin. Both these motions manifest distinctive magnetic moments, together contributing to the magnetic behavior of a material.

- Based on magnetic behavior, all materials can be classified into five distinct categories depending on their bulk magnetic susceptibility. The two most common variations of magnetism are diamagnetism and paramagnetism, accounting for the magnetic properties of most of the periodic table elements at room temperature (RT). The magnetic effect in materials is also understood by its prevalence in isolated elements or compounds. Going by this logic, the magnetically sensitive elements are included under the category ferromagnetic. Antiferromagnetism is another form of magnetic behavior noticed in pure elements at RT. Another magnetic effect is ferromagnetic, which is not at all observed in any pure element but is found only in pure compounds such as mixed oxides. Table 6 lists the different kinds of magnetic behavior in elements with suitable examples of each category
- In a diamagnetic material, the magnetic moments of constituent atoms are aligned to nullify each other's contribution in the absence of an applied magnetic field. However, as soon as an external magnetic field (H) is applied, the electrons undergo spinning motions, which generate an electrical current and ultimately an oppositely directed magnetization concerning that of the applied magnetic field. Readers must note here that all magnetically responsive materials exhibit a diamagnetic effect, but it often remains masked by greater paramagnetic or ferromagnetic sensitivities, which inevitably remain unaffected by the temperature.
- Multiple explanations and justifications are known for paramagnetism, holding distinctive validity for different kinds of materials. For instance, the behavior of materials characterized by noninteracting localized electrons is explained by ***Langevin model***, which works under the assumption that each atom has a randomly oriented magnetic moment due to thermal agitation. The application of a magnetic field modulates these randomly oriented magnetic moments, resulting in a gradual generation of low magnetization in a direction similar to that of the applied magnetic field. With a temperature rise, it becomes tedious to align these magnetic moments, resulting in decreased susceptibility. This kind of

response is recognized by "Curie's law" (Eq. 4), C being a material constant, also termed as "Curie's constant."

$$\chi = \frac{C}{T} \tag{4}$$

Materials obeying this law have their magnetic moments localized at the atomic or ionic sites with no interaction between neighboring magnetic moments. Equation 4 is a special case of generalized Curie–Weiss law (Eq. 5) having a temperature constant θ, which refers to the inclination at which a material is placed concerning the applied magnetic field.

$$\chi = \frac{C}{T - \theta} \tag{5}$$

It is important to note here that for a nonzero value of θ in Eq. 5 interaction happens between neighboring magnetic moments and the material is paramagnetic only above a certain transition temperature. The discussion here is restricted to diamagnetic and paramagnetic materials, and for other materials, readers are advised to refer the more specific literature sources (Issa et al. 2013).

- Pauli's model for paramagnetic behavior is deemed fit for materials having free electrons, deemed helpful in the interaction-driven formation of the conduction band. As per this model, conduction electrons are fundamentally free electrons that manifest an imbalance between the electrons with oppositely directed spin, generating a low magnetization in the direction of the applied magnetic field. The corresponding susceptibility of the material is not affected by the temperature until the electronic band structure is altered.

4.2.4 Biosensor Utilities of Magnetic Nanoparticles

- Utility attributes of MNPs for improved biosensing could be via their integration into transducer materials or dispersion within the sample followed by their control using an external magnetic field onto the active detection surface. Sensing mechanisms based on MNPs offer benefits in terms of analytical figures of merit such as enhanced sensitivity, low LOD, high SNR, and shorter analysis time compared to those for non-MNP-based strategies. Biosensors employing MNPs have been reported significantly for the detection of several analytes, distinguished via a linear range of detection and extremely low LOD extents. Table 7 provides a list of such biosensors working through distinctive transduction mechanisms such as electrochemical, optical, piezoelectric, and magnetic fields (Rocha-Santos 2014). The subsequent discussion of biosensor configurations of MNPs is exclusively compiled from the 2014 contribution by Rocha-Santos TAP, which was featured in *Trends in Analytical Chemistry*. So, readers are suggested to refer to this literature source unless the reference is otherwise stated.

Table 7 Salient performance aspects of MNPs-based biosensors, where the maximum utility of Fe_3O_4 in different configurations indicates its functional robustness with an ability of interaction-based modulated biological responses

Sensor distinction	Configuration of magnetic nanoparticles	Detection limit	Detection range	Analyte
Transduction principle: electrochemical				
Voltammetric immunosensor	Core–shell Au–Fe_3O_4	0.01 ng•mL^{-1}	(0.005–50) ng•mL^{-1}	Carcinoembryonic antigen
Voltammetric immunosensor	Fe_3O_4 Au NPs	0.22 ng•mL^{-1}	(10^{-3}- 10) ng•mL^{-1}	Clenbuterol (pork)
Voltammetric enzyme-based sensor	Au–Fe_3O_4 composite NPs	5.6 •10^{-4} ng•mL^{-1}	(10^{-3} to 10) ng•mL^{-1}	Organochloride pesticides (cabbage)
Voltammetric enzyme-based sensor	Fe_3O_4 Au NPs	2 •10^{-5} M	(2•10^{-5} to 2.5•10^{-3})M	H_2O_2 (contact lens care solution)
Voltammetric sensor	Core–shell Fe_3O_4@SiO_2	1.8•10^{-8} M	(5•$10^{-8}$to 10^{-6}) M	Metronidazole (milk, honey)
Voltammetric sensor	Fe_3O_4 anchored on reduced GO	ND	(0.2–0.6) nM	Cr (III)
Voltammetric sensor	Fe_3O_4@Au–MWCNT–chitosan	1.5 •10^{-9} mol•L^{-1}	(10^{-6} to 10^{-3}) mol•L^{-1}	Streptomycin
Voltammetric sensor	Core–shell Fe_3O_4@SiO_2/MWCNT	0.13 μM	(0.60–100) μM	Uric acid (blood serum, urine)
Amperometric enzyme biosensor	Core–shell Au–Fe_3O_4@SiO_2	0.001 mM	(0.05–1) mM/(1–8) mm	Glucose (human serum)
Amperometric enzyme biosensor	Fe_3O_4@SiO_2/MWCNT	800 nM	1 μM to 30 mM	Glucose (glucose solution)
Potentiometric immunosensor	Magnetic beads Dynabeads Protein G	0.007 μg•mL^{-1}	ND	Zearalenone (maize-certified baby food cereal, wheat, rice, maize, barley, oats, sorghum, rye, soya flour)

Potentiometric enzyme biosensor	Core–shell Fe_3O_4	0.5 μM	0.5 μM to 34 mM	Glucose (human serum)
Electrochemoluminescent immunosensor	Core–shell Fe_3O_4 @ Au NPs	0.2 pg•mL^{-1}	(0.0005–5) ng•mL^{-1}	α-Fetoprotein
Electrochemoluminescent immunosensor	Core–shell Fe_3O_4 @ Au	0.25 ng•mL^{-1}	(0–6) ng•mL^{-1}	CrylAc (N/A)
Electrochemical impedance immunosensor	FeO carboxyl-modified MNPs	0.01 ng•mL^{-1}	(0.01–5) ng•mL^{-1}	Ochratoxin A (wine)
Electrochemical impedance biosensor	Fe@Au nanoparticles-2-aminoethanethiol functionalized graphene NPs	2•10^{-15} M	(10^{-4} to 10^{-8}) M	DNA (N/A)
Transduction principle: optical				
SPR immunosensor	MNPs (fluid MAG-ARA) with FeO core	0.45 pM	ND	β-Human chronic gonadotropin (N/A)
SPR immunosensor	Fe_3O_4 @ Au MNPs	0.65 ng•mL^{-1}	(1–200) ng•mL^{-1}	α-Fetoprotein
SPR immunosensor	Fe_3O_4 MNPs	0.017 nM	(0.27–27) nM	Thrombin (N/A)
SPR immunosensor	Fe_3O_4/Ag/Au MNPs	ND	(0.15–40) μg•mL^{-1}	Dog IgG
SPR immunosensor	Fe_3O_4–Au nanorods	ND	(0.15–40) μg•mL^{-1}	Goat IgM
SPR immunosensor	Core/shell Fe_3O_4/SiO_2	ND	(0.15–40) μg•mL^{-1}	Rabbit IgG
SPR immunosensor	Core/shell Fe_3O_4/Ag/SiO_2	ND	(1.25–20) μg•mL^{-1}	Rabbit IgG
SPR immunosensor	FeO–carboxyl-modified MNPs	0.94 ng•mL^{-1}	(0.30–20) μg•mL^{-1}	Ochratoxin A (wine)
Fluorescence immunosensor	Fe_3O_4	ND	(1–50) ng•mL^{-1}	*Escherichia coli* (N/A)

(continued)

Table 7 (continued)

Sensor distinction	Configuration of magnetic nanoparticles	Detection limit	Detection range	Analyte
Transduction principle: piezoelectric				
QCM immunosensor	FeO magnetic nanobeads	0.0128	(10^3 to 10^8) cfu•mL^{-1}	Avian influenza virus H5n1 (chicken tracheal swab)
QCM biosensor	FeO MNPs	ND	(0.001–100) ng•mL^{-1}	*D. desulfotomaculum* (N/A)
QCM immunosensor	Fe_3O_4@SiO_2	0.3	(0.001–5) ng•mL^{-1}	C-reactive protein (human serum)
Electrochemical QCM immunosensor	Core–shell Fe_3O_4@Au–MWCNT composites	0.3	ND	Myoglobin (human serum)
QCM immunosensor	FeO MNPs	53	ND	*E. coli* O157:H7 (milk)
Transduction principle: magnetic field				
Giant magnetoresistive immunosensor	Cubic FeCo NPs	83		Endoglin (human urine) (human serum)
Giant magnetoresistive immunosensor	Cubic FeCo NPs	ND	125 fM to 41.5 pM	Interleukin-6

Giant magnetoresistive sensor	FeO with PEG coating	8 Oe	ND	N/A
Magneto-optical fiber sensor	Fe_3O_4 NPs	592.8 pm•Oe^{-1}	ND	N/A
Magneto-optical fiber sensor	Fe_3O_4 in magnetic fluid	162.06 pm•mT^{-1}	ND	N/A
Superconducting quantum interference sensor	Carboxy-functionalized FeO NPs	1.3•10^6 cells	ND	MCF7/Her2-18 breast cancer cells (mice cells)
Hall sensor	Mn-doped ferrite ($MnFe_2O_4$)	ND	10 to 10^5 cells	Rare cells: MDA-MB-468 cancer cells (whole blood)
Hall sensor	Mn-doped ferrite ($MnFe_2O_4$)	ND	10 to 10^6 cells	*Staphylococcus aureus*, *Enterococcus faecalis*, and *Micrococcus luteus* (spiking cultured bacteria in liquid media)

4.2.5 Electrochemical Biosensors

- Electrochemical devises work through measuring the electric current variations (potential difference and impedance) in terms of interactions between electrode and analytes. The electrodes herein could be coated with thin layers of biochemical or biological materials for improved surface activity (Michalet et al. 2005). The sensors working on this principle exhibit the benefits of rapidity, high sensitivity, relatively less expenditure, and lot much simpler methods of miniaturization and operations. Owing to such performance and automation distinctions, these biosensors find significant utilities in clinical, environmental, biological, and pharmaceutical domains. Improvements in the working of EC devices by the use of MNPs have manifolded their application potential via remarkable signal amplification abilities. To make this possible, MNPs can be used in EC devices via contact mode configurations with electrode surfaces, enabling the transport of redox-sensitive species apart from thin film formation, on the electrode surface.
- To date, Fe_3O_4 is the most preferred material in use for EC biosensors, attributed majorly to its superparamagnetic behavior, compatibility with antibodies, and enzymes, along with preparation simplicity. A potential constraint with Fe_3O_4 is due to its magnetic dipole-conferred attraction and a large aspect ratio, causing its aggregation on being exposed to bioactive fluids. This concern could be resolved by functionalization, which improves its interaction behavior and can make it biocompatible also. The salient functionalized architecture of MNPs in use for biosensors is core–shell Au–Fe_3O_4, core–shell Au–Fe_3O_4@SiO_2, Au–Fe_3O_4 composite NPs, Fe_3O_4 anchored on reduced graphene oxide, and Fe_3O_4@Au–MWCNT–chitosan (Dembski et al. 2008). Of these, core–shell Fe_3O_4@SiO_2 finds extensive use in biosensors as it allows MNPs' stabilization in the solution phase besides enhanced ligand binding on the MNPs' surface.

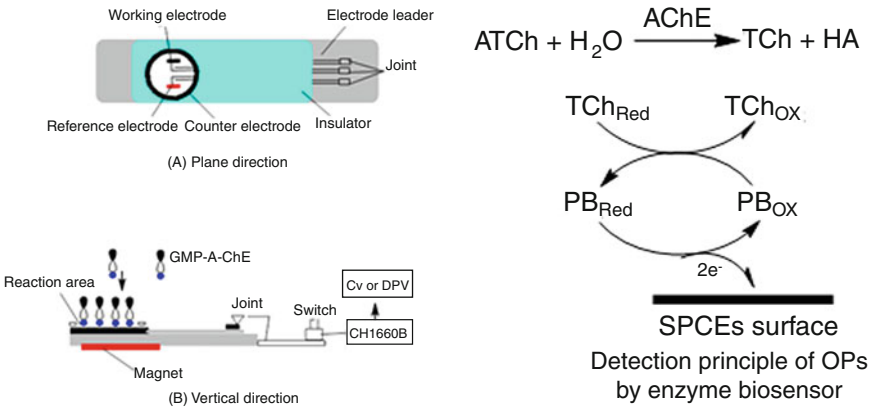


Fig. 17 Representative illustration of an electrochemical biosensor, apparatus view from (**a**) plane and (**b**) vertical directions. Adjacent is the detection principle for organophosphorus pesticide recognition

Next in line are the Au–Fe_3O_4-composite NPs, with their well-suited morphological, interactive, and surface attributes.

- In yet another worthwhile attempt, Gan and associates engineered a screen-printed carbon electrode (SPCE)-MNPs comprising composite. The working module of the sensor employed acetylcholinesterase (AchE)-coated Au–Fe_3O_4-composite NPs, which were adsorbed on the surface of CNT/nano-ZrO_2/Prussian blue/Nafion-modified SPCE (Fig. 17). Application of as-designed sensing probe for dimethoate detection in the cabbage produced an output equivalent to the gas chromatography coupled with flame photometer. The use of conducting Fe_3O_4/Au MNPs as sensing probes provided a faster response, adequate linear range performance, and sensitive detection of organophosphorus pesticides. Analysis with control combinations inferred a high electrode surface area and manifested a large current response for ultrasensitive thiocholine detection.
- A distinctive advantage of Fe_3O_4/Au MNPs-manifested thiocholine detection was the renewability of biosensor surface via removal of Fe_3O_4/Au/AChE through superparamagnetism of applied external magnetic field. Easier immobilization of enzyme/MNPs/(Fe_3O_4/Au/AChE) on SPCE helped in reducing manufacturing costs via inherent advantages of electrode integration, simple manipulation, low sample consumption, reduced use of expensive reagents, and simple experimental design. One exclusive attempt for MNPs-driven electrode regeneration along with reduced impedance using iron oxide carboxyl modified MNPs for anti-ochratoxin-A MAb immobilization. Using this advantage, Zamfir and colleagues fabricated an electrochemical-impedance immunosensor for ochratoxin-A detection using anti-ochratoxin-A MAb-iron oxide carboxyl-modified MNPs at the Au-constituted working electrode surface.
- Thus, MNPs can be easily gathered together as electrode surface entities to confer increased precision and adjustability of working, besides exhibiting the renewability of electrodes by releasing the MNPs and their reciprocated replacement.

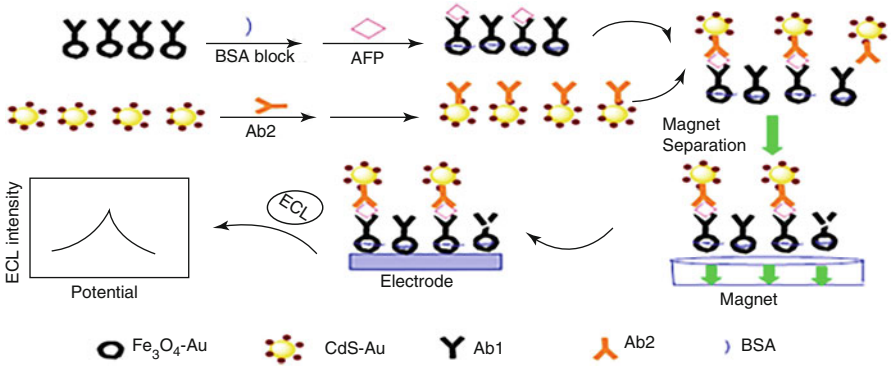


Fig. 18 Preparation procedure of an electrochemiluminescent (ECL) immunosensor. BSA, bovine serum albumin; AFP, α-fetoprotein; Ab1, primary antibody of AFP; Ab2, CdS–Au-labeled secondary antibody

So, the currently used electrochemiluminescent (ECL) biosensors used in functionalized mode with MNPs use the MNPs either as labeling agents or immobilization supports. The generated ECL signal is an amalgamation of sequential stages via chemical (radical combine) and ECL quanta (quantization of emitted energy). The ECL assays represent the workable arrangements of direct interaction, competition, and sandwich mode assays. Multiple QDs such as those of CdSe, CdS, or core–shell-type ZnS/CdSe have been the pillars of immense interest concerning ECL applications owing to the QC-aided optoelectronic applications. All these attributes make the QDs' efficient labels of ECL-based biosensors possessing improved sensitivity on being coated with MNPs and erstwhile capture probes.

- A significant 2012 attempt by Zhou and colleagues demonstrated the workability of a sandwich assay-based ECL immunosensor. The sensor detected α-fetoprotein using MNPs as capture probes and QDs as signal tags. Figure 18 illustrates the optimized process for making the magnetically sensitive capture probes, having Fe_3O_4–Au/primary α-fetoprotein (AFP) antibody (Ab1) and signal tag of CdS-Au/secondary AFP antibody (Ab2). The working configuration of the designed biosensor involved anchoring of Ab1 on the surface of Ab2, initiated by anchoring of Ab1 on the surface of Fe_3O_4–Au nanospheres through the Au–S linkage. As-formed products with Ab1 were immobilized on the Fe_3O_4–Au surface through which AFP was captured from the solution phase. Finally, protein-conjugated CdS–Au NPs were subjected to immunoreactions with the exposed AFP domain. The Fe_3O_4–Au/Ab1/AFP/Ab2/CdS–Au was used to fabricate the ECL immunosensor, which functioned via no signal response from the Fe_3O_4 MNP-modified electrode in the solution phase. However, Fe_3O_4–Au MNP-modified electrode surface generated a slightly enhanced ECL response.
- The introduction of CdS–Au as a label enhanced the immunosensor signal even further compared to that of the unlabeled system (Fe_3O_4–Au/Ab1/AFP). Additionally, 2.5 times enhanced ECL signal was noticed when the CdS–Au composite film was used instead of CdS NPs. This improved response with composite film could be attributed to the catalytic activity of Au NPs, which manifested in enhanced electrical conductivity and sensitivity. The immunosensor revealed a performance comparable with that of ELISA for the detection of AFP in human serum as a potential clinical application.

4.2.6 Optical Biosensors

- Optically sensitive biosensors have been used for the detection of multiple analytes of clinical, environment, and food background, attributed to their virtues of low SNR, reduced interferences, and moderate manufacturing costs. Optically modulated biosensors can be classified based on specific optical characterization, including fluorescence spectroscopy, interferometry, reflectance, chemoluminescence (CL), light scattering, and refractive index.
- The assays based on CL sensitivity require optimization concerning emission intensity and better selectivity for quantitative screening of biological and

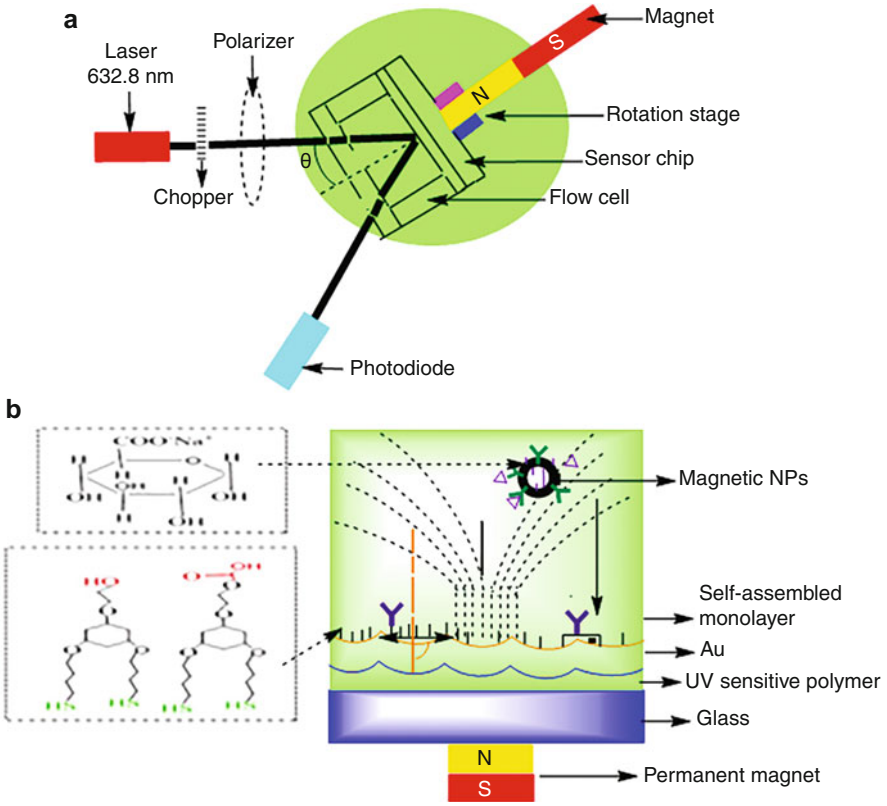


Fig. 19 Illustration of a surface plasmon resonance (SPR) immunosensor: (**a**) optical sensor setup and (**b**) a sensor chip of the MNP enhanced grafting-coupled SPR sensor

environmental stimuli. These constraints could be overcome using MNPs, wherein significant improvements could be accomplished in catalysis, biomolecules carrier, and separation accessory. Readers are suggested to go through the recent review article by Iranifam for a specific idea about analytical applications of CL detection in assistance with MNPs.

- The extensively primed detection mechanisms in the MNPs-based optical devices are SPR and fluorescence spectroscopy. Figure 19 depicts the functioning of the immunosensor in combination with SPR technology using MNPs assays equipped for the detection and manipulation of β-human chorionic gonadotropin (β-HCG).
- The underlying principle of this screening relies on a grafting-coupled SPR sensor chip that is functionalized with Abs recognizing β-HCG. The MNPs conjugated with Abs endowed dual advantages of labels intended to manifest enhanced refractive index changes after analyte capture as well as prompt analyte delivery carriers at the sensor surface.

- Capturing of MNPs-conjugated antibody and analyte was ensured via coupling with a magnetic field on the surface of a sensor. The use of MNPs and their manifested response on the surface of the sensor via the application of a magnetic field enhanced the sensitivity by four orders compared to that of regular SPR amidst the direct detection. The manifested enhancement was the outcome of larger mass and high refractive index of MNPs, producing an LOD of 0.45 pM corresponding to β-HCG detection. Observations of this investigation present a ray of hope for the detection of viral and bacterial pathogens, overcoming the low sensitivity of SPR-driven biosensors due to a formidable mass transfer hindrance on the sensor surface caused due to analyte diffusion.
- MNPs like Fe_3O_4 NPs could also enhance the analytical signal of fluorescence intensity. Using this principle, a microfluidic immunosensor chip having circular microchannels was developed for *Escherichia coli* detection. The methodology comprised Ab conjugation of Fe_3O_4 MNPs followed by in-flow antigen capture in the microchannels. The captured MNPs create a heap-like structure at the detection site under the influence of a reversed magnetic flow. This reversed magnetic flow increased the retention time of antigens at the site of capture and the capture efficacy of antigens, resulting in the enhanced intensity of the fluorescence signal.

4.2.7 Piezoelectric Biosensors

- Piezoelectric devices are driven either via quartz-crystal microbalance (QCM) or SAW oscillations. Table 7 depicts that MNPs-based piezoelectric sensors and biosensors work via QCM transduction. QCM is a quartz crystal disk with metal electrodes on each side that vibrates under an electric field influence. The frequency of this vibration depends on disk thickness and cut extent, wherein resonance is attained differently as compound(s) absorb or desorb from a crystal

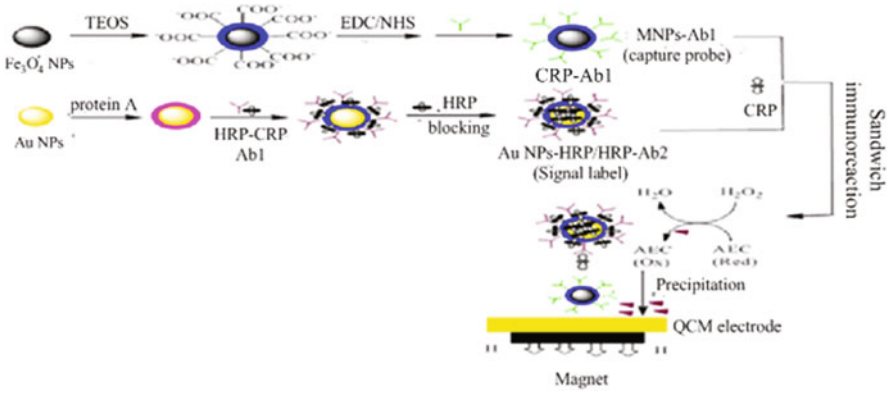


Fig. 20 Illustration of a quartz-crystal microbalance (QCM) immunosensor (up). Detection principle (down). TEOS, tetraethyl orthosilicate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, amine reactive *N*-hydroxysuccinimide; CRP, C-reactive protein; Ab1, primary CRP antibody; Ab2, secondary CRP antibody; Au NPs, gold nanoparticles; HRP, horseradish peroxidase; AEC, 3-amino-9-ethylcarbazole; MNP, $Fe_3O_4@SiO_2$ nanoparticle

surface. Reduction in frequency corresponds to the proportional mass of the adsorbed compound.

- QCMs are small, robust, and inexpensive provisions, equipped with producing significantly detectable aftermath corresponding to 1 ng mass change. A significant concern with these devices is the enhanced noise generation as the size reduces erupting from the increase in surface-to-volume ratio. Among other concerns of QCMs are the perturbations from atmospheric humidity along with the challenges for analyte screening within a solution.
- MNPs with piezoelectric attributes could easily overcome the above concerns owing to their incentive offering transduction activities and recognition with an ability of solid-state construction and cost-effectiveness. The mechanisms by which MNPs enable the enhancement in frequency include (1) inherent piezoelectricity of MNPs, (2) MNPs' binding and concentration of analytes at the QCM surface, and (3) actions as matrix carriers for label loading.
- A significant 2013 study demonstrated the working module of a QCM-triggered immunosensor, optimized for the detection of C-reactive protein (CRP). Workable mode involves a sandwich mode immunological reaction between SiO_2-coated Fe_3O_4 NPs (as capture probes) labeled with MNPs-CRP Ab1 (primary CRP antibody), CRP, and signal tag (HRP) conjugated with HRP-bound secondary antibody. The whole assembly is immobilized on Au NPs, leading to the workable configuration as: (Au NPs-HRP/HRP-CRPAb2). Thereafter, the generated immunological complex is subjected to 3-amino-9-ethylcarbazole (AEC) and hydrogen peroxide (H_2O_2).
- Figure 20 depicts the synthesis procedure with the detection principle, wherein the capture probe of MNPs-CRPAb1 enhances the analytical signal due to magnetic separation and immobilization at the electrode surface. The magnetic beads (Fe_3O_4-coupled SiO_2) for labeling CRPAb1 facilitated the attainment of uniform size distribution and simplified synthesis of labeled conjugates. In terms of results, the QCM methodology remained comparable with the ELISA, intended for human serum CRP detection. A further benefit is the easier regeneration of the QCM-sensor surface along with repeated usage. The present research aims to know more about the development of magnetic nanostructures along with understanding driven characterization of their piezoelectric behavior as these materials are capable of overseeding the accompanied sensitivity and stability concerns.

4.2.8 Magnetic Field-Based Biosensors

- As also reflected in Table 7, magnetically attuned transduction mechanisms involving MNPs' work based on giant magnetoresistance (GMR), Hall effect, magneto-optical, and superconducting quantum interference mechanisms. Magnetoresistive sensors work through magnetoresistance of either a ferromagnetic material or nonmagnetic heterostructures. Varying as per the compositional makeup of thin nanofilm, the MNPs exert their nanoscale effects using GMR or via tunneling magnetoresistance. The response signal generated out of changing electrical resistance regulates the quantitatively triggered analyte binding in the

presence of an external magnetic field. Changes in the signaling outputs could be monitored via screening infinitesimal variations in the magnetic field, having an explicit dependence on the applied magnetic field strength (in sensor surroundings).

- Two mechanisms are established for GMR and MNPs' aided interleukin-6 (IL-6) detection, the first characterized by functionalization of GMR sensor using capture antibodies after analyte and capturing antibody binding. The detection is monitored by the binding of MNPs' labeled antibodies with the perceived analyte. The second detection mechanism works via GMR sensor functionalization using capture antibodies, succeeded by GMR biosensor-screened binding of MNP-labeled analyte.
- Both configurations involved the MNPs' dipole field detection generated by sensor surface-captured MNPs, varying sensitively with the distance. The quality of MNPs is highly critical for accurate magnetoresistive detection, mandating the probes to be ideally superparamagnetic with a high magnetic moment and large susceptibility. This is required to ensure magnetization over a small magnetic field and the used MNPs are ideally needed to have uniform size and shape. This is because the magnetic response is decided by these features, which in turn regulate the corresponding stability in physiological solutions along with needful biomolecule coupling.
- Considering the above requirements, the MNPs are selected with high magnetic moment fetching enhanced signal and therefore a high sensitivity. Amidst this consideration for magnetoresistive detection, the preferred materials include metallic Fe, Co, or their alloy-based MNPs. In a study, Li and colleagues have claimed the coupling of a small NP volume with an applied field of 10 Oesterds (Oe), wherein the net magnetic moment of a single FeCo NP is almost 7- to 11-fold higher than one Fe_3O_4 NP.
- Controlling the magnetic moment of a permanent magnet using an externally applied nonhomogeneous magnetic field alongside the detection using magnetosensitive sensors is a potential domain where MNPs are highly useful. The best explanations of such phenomenon are provided by the magnetoresistive, Hall effect through which distinct variations of microscale magnetic field operable devices are controlled. This paved the way for the development of a micro-Hall sensor for ex vivo quantification of rare cells. The chip-based microfluidic sensor determines the magnetic moments of cells in a flowing configuration, labeled with MNPs. The workable sensor module acts via integrated technological breakthroughs, accomplishing biomarker actions on the implicit cells, like, as (1) linear response-mediated performance at >0.1 T, facilitating efficient magnetization of MNPs corresponding to maximal signal strength, (2) cell-equivalent dimensions of Hall element fetching an increased working accuracy, (3) an integrated setup of eight sensors consisting of a micro-Hall sensor with a moderated fluidic control than needed to focus the cells over a single sensor, and (4) an array that combines each cell's magnetic flux to facilitate the determination of the total magnetic moment of a single cell. This micro-Hall sensor is capable of high-throughput screening, exhibiting clinical-scale

significance using tumor detection from whole blood in 20 ovarian cancer sufferers, presumably at a higher sensitivity compared to that of clinical standards.

- A sensor working through the varied magnetic field was developed via integrating a magnetic fluid (Fe_3O_4 NPs) with an optical fiber Loyt–Sagnac interferometer. This sensor works via varied magnified birefringence of a magnetic fluid using an adequately configured optical fiber interferometer design. The sensitivity of this configuration was one to three orders greater than prevailing magnetic fluid sensors.
- Magnetic field-based biosensors are not well-acquainted for sensing the multiple analytes because the corresponding analyte response arises from a singular physical stimulus (the magnetic moment). Thereby, using the varied dimensions of superparamagnetic NPs, analyte responses could be distinguished via explicit magnetization curves, paving the way for simultaneous multiple analyte screening.
- Table 7 comprises the varying sensing configurations using MNPs, distinguished via electrochemical, optical, piezoelectric, and magnetic field-activated transduction mechanisms. It is evident from the compiled configurations that Fe_3O_4 NPs find a maximum utility significance owing to their nanoscale-enhancing coupling with Au and SiO_2 NPs in distinctive architectures. For instance, core–shell morphology safeguards the magnetic coupling of shell material by conjugating the magnetic effect of the core material. The Fe with an atomic number of 26 forms Fe_2O_3 (maghemite) and Fe_3O_4 (magnetite) oxides as its major oxidation products. However, in the sensing domain, Fe_3O_4 finds exclusively high utility as inferred from its workable combination with Au NPs, doping combinations with Mn, and immobilized state on multiwalled carbon nanotubes (MWCNTs) and in magnetic fluid too.
- With an outermost electronic configuration of $3d^6 4s^2$, Fe is ferromagnetic but its oxides, magnetite and maghemite, are ferromagnetic. Positioned in the 4th period and 8th group of the periodic table, Fe couples well with its neighbors, Mn and Co, for its magnetic susceptibility. Both Fe (alone) and its oxides exhibit a high magnetic susceptibility at less than critical temperature (T_c), arguing well for its structurally compatible combinatorial functioning. With both Mn and Co having an unpaired electron in their outermost shell, their combination with Fe argues for a magnetically coupled response through sustained electromagnetic induction.
- Spin behavior or alignment is the fundamental genesis of magnetic effect, wherein MNPs efficiently couple the magnetic sensitivity owing to dominant quantum effects in a sustained combinatorial regime with minimal adverse impact on native magnetic features of Fe or its oxide. The detection limit in most cases is in the ng•mL^{-1} range or in the order of 10^{-6} to even lower extents. Similarly, the detection ranges are in the order of μM, nM, or ng•mL^{-1}, together reflecting a high degree of functional sensitivity and suitability.
- The healthcare utility of developed sensing mechanisms is also well-inferred by the diversity of screened analytes, such as uric acid from blood serum or urine, metronidazole from milk or honey, organochloride pesticides (in cabbage), CRP,

endoglin (both in human serum), interleukin-6, *E. coli* contamination in milk, and avian influenza virus (H5N1). So, the diversity of these analytes is a strong indication of MNP's enhanced sensing abilities for screening more than one troubling pathological disturbance.

- With the coupling of the external magnetic field, MNPs-driven biosensors exhibit an added incentive in their external control and site-directed activities with greater accuracy. These attributes have been significantly helpful in coupling the localized thermomagnetic influences, enabling efficient eradication of tumor cells through controlled site-directed delivery. The magnetic coupling from an external agency is a handy manifestation in the removal of administered MNPs after their designated in vivo activities.

4.3 Reusable Substrate–Analyte Interactive Platforms

Enzymes are the heart of a biosensor working, wherein the usage for large extent commercialization assumes restriction because of high costs, which make the sensing operation highly expensive because of rather the low reusability of enzymes. Although NMs do require a minimum need of enzymes but ideally do not bypass it completely. The structural suitability of most enzymes during any biochemical reaction is very challenging, owing to which immobilization of useful enzymes with functional efficacy and enhanced reproducibility is practically highly desired even though it contributes to additional expenditure. The following are some important aspects regarding the immobilization of enzymes for their biosensing attributes:

- Enzymes, along with whole cells, can be used squarely well for immobilization. To put it more simply, immobilization of enzymes is a technique intended for their confinement over a solid matrix/support distinct from the one possessing any traces of the substrate or product. The simplest way to accomplish this is via attachment of enzymes over a suitable support matrix. Herein, the substrate molecules and the generated products must move in and out of the phase without any restriction. The phase here refers to the one in which enzymes are restrained.
- Multiple materials are suitable for use as matrix or support to aid in immobilizing the enzymes that facilitates a 100% recovery and reuse to aid in their cost-effective use. Usually, inert polymers and inorganic materials are preferred as enzyme support matrices. The major characteristics of an industrially viable carrier matrix are (1) cost-effective, (2) chemically stable, (3) physically compatible for handling and storage, (4) capable of augmenting enzyme specificity, (5) reusability, (6) effective for moderating product inhibition, and (7) forbidding of random adsorption and bacterial adulteration.
- In general, most of the matrices used exhibit only a few of these attributes, owing to which one has to optimize the performance features under the working conditions for the selection of an enzyme immobilization matrix. Typical enzyme immobilization is characterized by continuous economic operations,

automatically controlled unit operations, large-scale investment, and viable product recovery.

The most used enzyme immobilization methods are as follows:

- Adsorption/carrier binding
- Covalent binding/cross-linking
- Entrapment method
- Membrane confinement

The following points discuss these mechanisms concerning their practical feasibility and conduct. Most of the information is reported after taking inputs from a 2016 literature source by Sirisha and colleagues, featured in *Advances in Food and Nutrition Research*. So, unless otherwise stated, the readers are requested to refer to this article for recalled studies and observations as there is a legal restriction about including fewer references.

4.3.1 Adsorption/Carrier-Binding Method

- This method of enzyme immobilization keeps the enzymes adhered to a carrier matrix through a mix of hydrophobic proximities and the multiple salt linkages corresponding to each enzyme molecule. The process uses either matrix suspension with the enzyme or dried enzyme(s) on the electrode surfaces. Commonly used matrix materials are water-soluble carriers like polysaccharide derivatives, glass, synthetic polymers, etc.
- Characteristic interactions of this enzyme immobilization method are mitigated by a strong bond configuration between the enzyme and support milieu. Essential physiological parameters affecting the explicit interactions are temperature, pH, and substrate inclusion, excess of which can weaken the enzyme-support binding. Adsorbed enzymes are, in general, resistant to proteolysis and aggregation due to interfacial hydrophobic interactions. Certain attributes of suited matrix materials include micro-/mesoporous entities having high surface areas, thiol group functionalization, and microcrystalline cellulose with irreversible chemical binding.
- The prevalence of silanols makes the silanized molecular sieves suitable for enzyme immobilization stabilized via hydrogen bonding. Immobilization efficacy can be improvised by certain chemical modifications of functional support matrices. In this context, a study by Persson and associates screened the efficacy of adsorbed lipase over the polypropylene-grade hydrophobic granules/Accurel EP-100 apart from analysis of lipases–water chemistry. Interestingly, reducing the particle size of Accrual caused a significant increment in the enantiomeric stoichiometry and corresponding reaction pace.
- Physical adsorption-mediated immobilization of *Yarrowiali political* lipase on octyl agarose and octadecyl-sepa beads is a method of high stability, higher yield, and much greater operational regulation with low expenditure contrary to using free lipase. The distinctions in the immobilization-assisted lipase use are

attributed to hydrophobic interactions of octadecyl-sepa beads, resulting in enhanced enzyme-support matrix affinity.

- Similarly, adsorption of lipase from *Candida rugosa* over the poly (3-hydroxybutyrate-ω hydroxyvalerate) revealed 94% activity despite 4-h treatment at 50 °C and reusability for 12 cycles. In another significant study, Mishra and colleagues reported enhanced pH stability with 50% retainment for urease by adsorbing over the 1,4-butanediol diglycidyl ether activated by threads, in the dried state. The highlighted aspects elucidate the significance of eco-friendly support materials as a biodegradable, long-lasting, and biocompatible performance, illustrating suitability for steadfast use with no serious ethical concerns.

4.3.2 Covalent Binding

- This mode of enzyme immobilization exhaustively depends on the formation of covalent linkages between the enzyme and support matrix, involving the side chain comprising histidine, arginine, and aspartic acid. However, the reactivity is indeed influenced by the orientation of various functional groups like those of ***carboxylic, phenolic, indole, amino, thiol, imidazole, sulfhydryl, and hydroxyl*** functionalities. Enhanced enzymatic actions could be accomplished via ensuring null inactivation of active-site amino acid residues. Some of the widely used enzyme-immobilizing modes based on covalent binding are as follows:
  - Enzyme–matrix covalent interaction in the presence of a substrate or a competitive inhibitor.
  - Formation of the reversible covalently linked enzyme–inhibitor complex.
  - Presence of a zymogen receptor.
- Usually, much greater activity with high stability is noticed corresponding to a characteristic protein orientation *vis-à-vis* involvement of peptide-engineered faces enzyme conjugation. Observations of a study are quite significant in this regard, whereby greater thermal stability of the enzyme was observed on its covalent linkage with cyanogens bromide (CNBr)-conjugated agarose, CNBr-sepharose, with carbohydrate moiety, and glutaraldehyde as spacer arm. Furthermore, covalently bonded enzymes with modified silica gel carriers (on eliminating nonreactive aldehyde groups with SBA-15 matrix) impart enhanced stability and make them suited to function as hyperactive biocatalysts.
- Other matrix materials popular in use for covalent linkage-driven enzyme immobilization include mesoporous silica and chitosan, well suited to enhance the half-life and improve the thermal labile functioning of enzymes. Using the nanoscale materials (such as nanosheets, NPs, carbon nanotube(s) functionalization) is a revolutionary attempt in improving enzyme functioning. Studies have demonstrated that cross-linking of enzymes with electrospun nanofibers confers a much higher residual chemical activity due to enhanced porosity and surface area (highly important for reducing the activation energy of reactants). Researchers in this context have obtained significant performance improvement using attapulgite nanofibers as support matrices for covalently linked alcohol dehydrogenase, attributed to its thermal flexibility and enhanced quantum effects at the nanoscale.

- Another prominent route to accomplish covalent linkage-driven enzyme immobilization uses magnetic nanoclusters, which operate in multiple directions and are the crux of remarkable improvement in the pharmaceutical industry. The salient attributes of these materials are enhanced operational stability, reuse ability, and longevity of operation. A distinct aspect of this method is the involvement of a cross-linking agent, which is glutaraldehyde. The high aqueous solubility of this material enables the formation of stable intra- and inter-subunit-linked covalent bonds, eminently referred to as bifunctional cross-linkages.

4.3.3 Affinity Immobilization

- Affinity-binding-driven enzyme immobilization over a support matrix is stabilized via specific BFs. This method uses the substrate-specific action of an enzyme to engineer the support matrix in varied physiological conditions. The following points discuss the two methods used to accomplish this:
- The first approach is characterized by pyrimidine coupling of the matrix to an affinity-driven ligand corresponding to the target enzyme. The second method uses enzymes in the conjugated phase with another molecule, gradually developing affinity toward the matrix.
- The affinity adsorbents also find significance in enzyme immobilization, wherein immobilized enzymes on complex support matrices like agarose-linked multilayered concanavalin A and alkali-inert chitosan-coated porous silica beads exhibit increased efficacy and stability.
- The bioaffinity-assisted layering of matrices is another potential method used to modulate the reusability of bound enzymes. The inherent benefit of this modulation is due to noncovalent interactive forces, such as van der Waals forces, Columbic interactions, and hydrogen bonding.

4.3.4 Entrapment

- The entrapment process relies on enzymatic caging within a polymeric system via covalent or noncovalent bonds, facilitating the substrate and product movement but retaining the enzyme. A distinction from the other methods is the nonbound state of the enzyme to the support matrix.
- Enzymatic caging could be accomplished by (1) inclusion within a highly cross-linked polymer matrix, (2) dissolution in the nonaqueous phase, and (3) semipermeable capsule-driven separation from a bulk solution.
- Various approaches to enzyme entrapment are known like fiber entrapping, gel entrapping, and microencapsulation. Encapsulation with **alginate–gelatin–calcium hybrid carriers** is reported as efficient due to its ability to improve mechanical stability and prevent enzyme leakage. In *C. rugosa*, the entrapping of lipase in chitosan resulted in its enhanced activity besides forbidden leaching.
- Such abilities are chiefly attributed to the biocompatible and nontoxic features of the support matrix, which is receptive to chemical modifications and has hydrophilicity-driven high affinity toward affinity. Chen and colleagues in 2011 reported simultaneous entrapment of lipase and MNPs with biomimetic silica,

conferring a significant improvement in enzyme activity under various saline additives.
- Studies have also reported that on being entrapped with K-carrageenan lipases exhibit high tolerance toward organic solvents alongside being highly thermostable. The use of nanostructured supports like electrospun nanofibers and pristine materials for enzyme entrapment is witnessing a persistent encouragement for biomedicine, biofuels, chemistry, and biosensors.

4.3.5 Ionic Bonding

- This method binds the enzymatic protein to hydrophobic carriers, containing ion-exchange residues. The typical mechanism is characterized by noncovalent reversible interactions via changes in ionic strength, polarity, and temperature. The working principle herein is similar to the functional protein–ligand interactions in chromatography.
- Support materials used in this enzyme immobilization module include polysaccharides and synthetic polymers, possessing ion-exchange domains. Advantages of this method comprise simpler and easier binding of the enzyme with the carrier unlike that of covalent binding. Usually, ionic binding induces little changes in the confirmation and active-site constitution, enhancing the enzymatic activity on most occasions.
- A cautionary aspect herein is the supposed enzyme leakage from the carrier, particularly if high ionic strength substrate or varied pH solutions are used. This is due to the weak BFs between the enzyme proteins and carrier molecules. The enzyme-carrier BFs are quite strong in this mode contrary to that of physical adsorption, although these are not as strong as in covalent binding.

4.3.6 Metal-Linked Immobilization

- This method of enzyme immobilization relies on the precipitation of metal salts over the support matrix. The metals suited for this purpose are capable of binding with nucleophilic functionalities of the carrier matrix. Precipitation of constituent cations over the support matrix surface is attained on heating.
- Studies have demonstrated (30–80)% improved enzymatic activity on immobilization via this method. The mechanism of this process is relatively simple and reversible in its operation. A distinctive benefit of this method is that it allows the regeneration of both enzymes and a matrix.

4.3.7 Preferable Materials for Designing Immobilization Supports

The materials employed for the immobilization of enzymes are frequently referred to as a carrier or support matrices. The ideal features of a matrix are of substantial process in accomplishing a requisite extent of enzyme immobilization. Ideal support matrices should have the following attributes for optimal performance:

1. It should not be much costly and must be eco-friendly to get rid of the undesired economic impact of the process.

Table 8 Eminent inorganic supports with their characteristic attributes and immobilized enzymes

Inorganic material	Traits	Immobilized enzyme
Zeolite (molecular sieves)	Large specific surface area conferring efficient enzyme loading	Glucose oxidase (GO), α-chymotrypsin
Ceramics	Both macro- and micropores are efficient in moderating diffusion rate and enhancing the specific surface area	*Candida antarctica* lipase
Celite	Inexpensive, less polar, large adhesion area, chemically inert, resistant against pH, temperature, urea, detergents, and solvents	Lipase, polyphenol oxidase, β-galactosidase
Silica	Nanosized structures have a high surface area, crystallinity, and high stability to chemical and mechanical forces	Lignin peroxidase, HRP, α-amylase
Glass	Highly viscous fluid	α-Amylase, nitrate reductase
Activated carbon	Manifold contact sides, high surface area	Acid protease, acidic lipase
Charcoal	Splendid adsorbent, least release of fine particulate matter	Papain, amyloglucosidase

Note: Large surface area, robust handling, and chemical stability are the major distinctions

2. It must be entirely inert after immobilization and should not block the desired reaction.
3. Adequate thermal and mechanical resistance to ensure the use of immobilized enzymes under varied operational conditions.
4. The matrix constituent must be significantly stable.
5. The matrix material should have high renewability after the attainment of the useful lifestyle of the immobilized enzyme.
6. The chosen matrix material must enhance the enzyme specificity.
7. The chosen material should be capable of packing a large amount of enzyme. For this ability, porosity plays a significant role. In general, a large pore size leads to a significant decrement in surface area contrary to a difficult exclusion of the protein with a small pore size. So, the pore diameter must be within the optimal range.
8. To get rid of inhibiting protein adsorption and denaturation, the hydrophobicity of the support matrix must be reduced. Thereby, the support matrix working environment should be amenable to an extent that favors the catalytic activities of immobilized enzymes.
9. The support matrix should be capable of shifting the pH optimum for accomplishing the desired extent of enzyme action.
10. The support matrix should have antimicrobial and nonspecific adsorption characteristics.

11. Rarely ever a matrix is available with an optimum extent of each of the above properties, so caution must be thoroughly exercised in choosing an appropriate support matrix *vis-a-vis* their properties.

Based on their chemical compositions, the support matrices are listed in two major categories, namely, (1) inorganic support material and (2) organic support material (organic supports are further divided into natural and synthetic materials). The following points discuss the salient aspects of these entities with suitable examples.

4.3.8 Inorganic Materials as Supports

- The materials included in this class include glass, silica gel, alumina, metal oxides, zirconia, and several other silica-based compounds that are ideal materials and are widely used due to their significant thermal and mechanical resistance. These entities also exhibit significant microbial opposition for inhibited bacterial or fungal growth due to their substrate attributes.
- Rigidity and porosity are the two characteristic features of inorganic support. Besides, these materials exhibit unchanged ore diameter and volume ensuring a fixed volume and shape of the support. Table 8 comprises several inorganic support matrices with their major characteristics and extensively immobilized enzymes.
- **Silica**: Inorganic supports, like SiO_2 and SiO_4, constituted matrices are highly suited for enzyme immobilization. Both of these prevail as 3D polymers, although SiO_4 is rigid while SiO_2 is flexible. In both these support matrices, amphiphilic functionalities contribute to the adsorption efficacy. Zeolites comprise another domain of silica-rich microporous matrix surfaces, equipped with enhanced surface area to immobilize the proteins. Though silica carriers are relatively inert materials, these require significant optimization for being used as an enzyme immobilization support matrix. For this purpose, the silica bed supports are preferentially treated with aminoalkyltriethoxy silane that engineers the matrix surface with $–NH_2$ functionalization. A series of further modifications make these materials befitting for enzyme immobilization. For example, penicillin-G-amidase is initially conjugated with dextran before its immobilization over the amino-activated silica gel that confers its enhanced thermal stability. Similarly, lignin peroxidase and HRP were immobilized on activated silica to get rid of chlorolignins from the eucalyptus kraft effluent. In a significant attempt, Soleimani and colleagues observed an improved cleansing action of detergents after their binding with α-amylase on silica NPs. Another study reported enhanced enzyme carrier bonding after surface engineering of silica, involving amination of –OH and siloxane functionalities apart from absorption of methyl or polyvinyl functionalities.
- **Ceramics**: These entities are solid, insoluble, and nonmetallic and are derived from cooking plastic materials. **Metal oxides such as Al_2O_3, TiO_2, ZrO_2, and SnO_2 are referred to as ceramics**. Due to their porous character, ceramics are highly preferred materials for enzyme restriction. A 2004 study by Magnan and

Table 9 The various natural organic polymeric supports used for enzyme immobilization

Support	Properties	Enzymes immobilized
Alginate	Used in the form of xanthan-alginate beads, alginate-polyacrylamide gels, and calcium-alginate beads Can be reused and significantly improves enzyme stability	Cellulase, cross-linking with divalent ions and glutaraldehyde stabilizes the enzyme activity
Chitosan and Chitin	Used in combination with alginate, less leaching, reliable for enzyme trapping, and can entrap more enzymes as beads	D-Hydantoinase, acid phosphatase, chymotrypsin, glucose isomerize, glucose oxidase, and lactase
Collagen	Retains significant activity after multiple reuse cycles	Tannase, catalase, and alkaline phosphatase
Carrageenan (linear sulfated polysaccharide)	Improves stability, pseudoplastic sensitivity imparts thinness under shear stress and recovers the corresponding viscosity on stress removal. Cheap, long-lasting, and has greater entrapment	Lipase and α-galactosidase
Gelatin (hydrocolloid rich in amino acid content)	High adsorption ability and promotes loading efficacy	Amylase, urease, and galactosidase via cross-linking with glutaraldehyde and formaldehyde
Cellulose	Greater storage capacity, better formability, and flexibility	Fungal laccase, penicillin G acylase, glucoamylase, α-amylase, tyrosinase, lipase, and β-galactosidase
Starch	Stable	Bitter gourd peroxidase, α-amylase, and glucoamylase
Pectin	Used in combination with glycerol as a plasticizer to reduce the brittleness of pectin-chitin and pectin-calcium alginate, have enhanced thermal, denaturant resistance, and catalytic attributes	Papain and pectin lyase
Sepharose	Porous, easy adsorption, retain catalytic properties at extremes of pH, temperature, and high salt concentration	Amylase and glucoamylase

colleagues reported lipase immobilization from *Candida antarctica* on a ceramic membrane that modulates hydrolytic and synthetic reactions through curtailing feedback inhibition. Ceramic foams were found effective in enhancing the working surface area and decreasing the diffusion rates.

- **Glass**: The support matrices of glass represent another prominent inorganic enzyme immobilization means. Glass-made matrices are highly viscous and have been successfully recruited as immobilized supports for enzymes like α-amylase. In the process, phthaloyl chloride comprising $–NH_2$ group-functionalized glass beads were screened as strong and sustainable. Likewise,

the reason being immobilized over the glass pH electrodes acted as a biosensor for monitoring blood urea extent to as low as 52 mg•mL^{-1}.

- **Charcoal**: The use of charcoal as immobilized support is immensely popular for enzymes like amyloglucosidase with no cross-linking agents for starch hydrolysis and accomplishing 90% catalytic activity. Charcoal has been studied as an efficient adsorbent with efficient adsorption ability and a low release of particulate matter.
- **Activated carbon**: Studies have shown reliable evidence for natural as well as HCL-modified activated carbon for enzyme immobilization. Recently, acid proteases and acid lipases were immobilized on mesoporous-activated carbon particles and demonstrated significantly greater catalytic efficacy even after being reused for 21 cycles (Sirisha et al. 2016). A 2010 study by Daoud and associates reported remarkable enzyme immobilization enabled via (600–1000) $m^2.g^{-1}$ surface area and (300–1000) pore volume.

4.3.9 Organic Supports

- **Natural polymers**: A wide range of natural materials, such as hydrophobic polysaccharides, viz., chitosan, collagen, carrageenans, alginate, cellulose, starch, agarose, etc., are widely preferred as support matrices for immobilization of enzymes. The structural flexibility of similar materials makes them capable of forming inert gels due to easily activated chemical functionalities. Such attributes facilitate a reversible and irreversibly optimized binding of proteins and enzymes over the support matrix. Other significant advantages of these natural-grade materials as immobilization matrices include their cost-effectiveness and thermal and mechanical inertness on being cross-linked with bifunctional chemical agents. Table 9 describes some widely used organic supports as enzyme immobilization support matrices.
- **Alginate**: This is a sulfate-functionalized polysaccharide obtained from the brown algal cell wall. Concerning the usefulness of alginate in enzyme immobilization, xanthan-alginate beads, calcium alginate beads, and alginate-polysaccharide gels are well known for improved enzyme performance and reusability. Improved enzyme stability via the use of alginate is well reported, involving cross-linking with divalent ions and glutaraldehyde.
- **Chitosan**: It is a polysaccharide that finds usability as a support matrix having manifold optimal carrier suitable characteristics. These attributes include mechanical inertness, rigidity, hydrophilicity, and active reactive groups that can directly interact with enzymes besides having an affinity for protein binding. Studies have reported that chitosan-coated enzymes exhibit less leaching compared to those of alginate due to chitosan's more prompt ionic and physical interactions with enzymes. It has also been demonstrated that a wet composite of chitosan and clay has plentiful –OH and $–NH_2$ groups. This material is thereby more efficient for enzyme immobilization with enhanced hydrophilicity and porosity. It was observed that *Bacillus circulans*, a chitin-binding domain of chitinase, confers a strong affinity for retaining D-hydantoins.

- **Cellulose**: This is perhaps the most commonly used carrier molecule for enzyme immobilization. It has a distinguishingly lower binding capacity besides being inexpensive and available in fibrous as well as globular textures. A notable study for enzyme immobilization using cellulose was reported by Namdeo and Bajpai in 2009, wherein α-amylase was immobilized to cellulose dialdehyde-coated magnetite NPs and enabled a novel starch degrading system. Likewise, enhanced forbearance and viability of the enzyme were obtained following its immobilization over the glutaraldehyde-activated ionic liquid-cellulose film.
- **Collagen**: The attributes of collagen making it an efficient enzyme immobilizing support matrix comprise protein similar nature, significant water-holding capacity, and good porosity. The basis of collagen immobilization is via the formation of a covalent bond between the collagen side chains and that of the enzyme, resulting in a stronger enzyme hold over the support. Using glutaraldehyde as a cross-linking agent, collagen is used for tannase immobilization. Chen and associates 2011 showed that on being immobilized to Fe^{+3}-collagen fibers, catalase activity remained significant even on 26 reuses.
- **Carrageenans**: It is a red algal and linear-sulfated polysaccharide. The attributes of carrageenans contributing toward its significance in enzyme immobilization include good gelling and high protein-holding ability with pseudoplastic receptivity, collectively making it thin under shear stress besides enabling a viscosity recovery on being distressed. A 2010 study by Jegannathan and colleagues demonstrated an encapsulation efficacy of 42% during biodiesel generation via the coextrusion method using carrageenans as a support matrix.
- **Starch**: A natural polymer with linear amylase and branched amylopectin units, for significant hydrophilic actions, starch is a renewable and one of the most trusted raw materials used for enzyme immobilization. In this context, a 2019 investigation by Matto and Hussain reported significant immobilization for bitter gourd peroxidase using calcium-alginate and starch comprising hybrid. Analysis revealed comparatively greater stability of the entrapped enzyme in the presence of urea (a denaturant) because of the sustained and coordinated chemical actions of carbohydrate moieties rather than that for the surface-immobilized enzymes (without the alginate-starch-calcium hybrid).
- **Pectin**: This is a structural polysaccharide of plant origin and is a major constituent of the primary cell wall. In plant tissues, pectin serves as a significant intercellular cementing material. It is a gelling agent with good water-holding capacity. Ceniceros and colleagues 2003 developed advanced materials for the treatment of skin damage through papain immobilization onto pectin along with (0.2–0.7)% glycerol. The ability to form highly stable polyelectrolyte complexes with entrapped enzymes makes pectin–chitin and pectin–calcium-alginate confers them with significant thermal and denaturant resistance for improved catalysis by the immobilized enzyme.
- **Sepharose**: This enzyme-supported matrix finds its commercial significance from its beaded texture and is activated by cyanogens bromide (CNBr). The CNBr-activated Sepharose 4B is used for the immobilization of amylase and glucoamylase, characterized by enhanced porosity with better and greater

macromolecule adsorption. Alkylation-engineered sepharose increases its catalytic retainment at extreme pH, high salt concentration, and temperature.

- **Artificial Polymers as Immobilization Support Matrices**.

  Polymers of synthetic grade comprise the ion-exchange resins, with a porous surface and a hydrophobic sensitivity. These characteristics impart a strong enzyme immobilization potential to synthetic polymers, exemplified by their inertness toward a microbial attack. Polystyrene, polyvinylchloride, polyacrylate, polyamide, polypropylene, diethylaminoethyl cellulose (DEAE cellulose), and UV-activated PEG are some prominent synthetic polymers used as enzyme support matrices. Polystyrene was the first-ever synthetic polymer used for enzyme immobilization.

Some other significant attempts using synthetic polymers for enzyme immobilization are summarized next:

1. Amberlite and DEAE cellulose for α-amylase immobilization.
2. PVC is used for immobilizing cyclodextrin glucosyltransferase and guards against thermal inactivation.
3. Synthetic polymer polyurethane microparticles were produced by mixing polyvinyl alcohol and hexamethyl diisothiocyanate in 1:3 proportions. The use of polyurethane microparticles for enzyme immobilization improved the loading ability and catalyzing efficiency through the enhanced surface area.
4. Polyethyleneglycol, along with glutaraldehyde, is used for immobilization of white radish peroxidase and forms a protective layer around the active enzyme center to guard against oxidative stress.
5. UV-activated PEG having high porosity has been used for wastewater treatment.
6. Glutaraldehyde-activated nylon is well known for lipase immobilization.

5 Early Disease Diagnosis Using a Biosensor

5.1 Mechanisms for Improved Detection Sensitivity

The present diagnosis of critically infectious diseases caused by bacterial, viral, or fungal pathogens relies on manifold laboratory-based tests, including microscopy, culture immunoassays, and nucleic acid amplification. Primitive methods for in vitro diagnosis rely on age-old mechanisms that are time-consuming and mandate the analysis in centralized laboratories, skilled manpower, and larger-sized equipment. With the progressive miniaturization and constantly bettered understanding of nanotechnology principles, the point-of-care diagnosis methods have been a boon to faster and more accurate diagnosis without cumbersome, elaborate laboratory testing and the manpower requirement. Biosensor-mediated diagnosis of infectious diseases is characterized by improved DS of pathogen-implicit antigens with the multiplex detection of host-immune response for an overall improved specificity.

The flexibility exhibited by a biosensor functioning augments the development of integrating the detection of pathogen-varied targets as well as typical biomarkers that represent distinctive host-immune responses at variable stages of an infection. Physical distinctions of biosensor-mediated clinical diagnosis of infectious diseases comprise a low requirement of to-be analyzed sample, a robust screening mechanism based on specific interactions, reduced cost, time, and user-friendly operation. Working in labeled or label-free configurations, the modern biosensors imbibe the integrated micro- and nanofabrication technologies, working through optical, electrical, and mechanical transducers. While label-free assays screen the analyte via biochemical reactions on a transducer surface, the labeled assays comprise the analyte sandwiching between capture and detector agents, wherein the latter is conjugated using enzymes, fluorophores, or QDs toward a quantifiable signal output. The following points discuss the two modes with some more illustrations.

- **Labeled assays**: These are the most commonly used biosensing assays, having the analyte sandwiched between the capture and detector agents (CAs and DAs). The CAs are immobilized on a solid surface such as electrodes, glass chips, and nano- or microparticles, whereas the DAs are conjugated to signaling tags that could be fluorophores, enzymes, or NPs.
- **Label-free assays**: These assays couple the optical, electrical, or mechanical transducers with the signaling tag. The sensor–tag interactions diversify the optical sensors equipped for perceiving the fluorescent, colorimetric, or luminescent tags (Sin et al. 2014).
- While electrochemical sensors are capable of detecting the redox reactions from enzyme tags, magnetoresistive sensors detect magnetic tags. Thus, together these systems enable quantitative or semiquantitative analyte detection by relating the generated signal with the captured analyte quantity. In general, capture and detector elements exhibit different binding sites, leading to enhanced specificity with reduced background. Some assays of labeled configuration are as described next.

Table 10 Working interfaces of labeled biosensors, with corresponding distinctions and possible concerns

Technology	Benefits	Uncertainties
Redox electrochemistry (amperometric)	Adjustable detection platform for POC configuration and easier integration with other electric field-driven approaches	No real-time detection and assay involves multiple steps
Bio-barcode	POC-compatible detection platform and easy-to-interpret results	No real-time detection, cumbersome probe preparation, and multiple steps in the assay
Metal nanoparticles	POC-compatible detection platform, easy-to-interpret results, and multiplex	No real-time detection, temperature fluctuation affected assay results, and multiple steps in the assay

POC point of care

- Practiced as the most standardized sandwich immunoassay for infectious disease diagnosis in clinical laboratories, enzyme-linked immunosorbent assay (ELISA) typically uses capture and detector antibody modified with an enzyme tag to facilitate the conversion of a chromogenic substrate to colored molecules. The quantitative configuration measures the optical density of the colored product and upon comparing it with a standard serial dilution of a known target molecule (the screened antigen) concentration. For instance, Liao and team developed an electrochemical sensor assay for the specific detection of urinary pathogens in clinical samples. The detection was facilitated by immobilized capture oligonucleotide and labeled detector oligonucleotides for screening for the existence of bacterial 16S rRNA. Signal was generated by a redox current of the conjugated enzyme tag to the detector probe.
- To date, the best-known commercial-grade sandwich assays are the lateral flow immunoassays (also referred to as immunochromatographic test strips), equipped with qualitative measurements of the signal by visual observation and on a semiquantitative mode via engineering interfaces such as low-cost laser photodiode or amperometric detectors.
- Some of the better commercial assays in terms of discrete analysis are the home pregnancy detection kits and urine analysis strips. Likewise, lateral flow assays are also well known for the analysis of saliva, HIV antigens, malaria, and tuberculosis screening using serum inspection. Salient benefits of lateral flow assays include moderate expenditure, almost very minimal sample preparation, and a robust interpretation of results (Sin et al. 2014). Demerits of this configuration involve a poor sensitivity to distinctly recognizing the clinically suited targets and the respective qualitative/semiquantitative results. Recent attempts have worked out the signal amplification through functionalizing the NPs (having a high aspect ratio) with several different kinds of biomolecules, significantly bettering the LOD. As of now, the latest design of labeled biosensors is characterized by the incorporation of bio-barcodes, metal, and magnetic NPs (Table 10).
- **Bio-barcode amplification**: This labeling procedure is amicable for detecting proteins and nucleic acids without any essential need for enzymatic reactions. The conventional procedure for bio-barcode amplification (BA) comprises a sandwich assay, with the capturing of targets using micro-/nanoscale particles conjugated with oligonucleotides as proximal signal amplification alternatives. Procedurally, on capturing each target, several barcode DNA strands are generated for the next detection using an energetic impulse (usually electrochemical or optical). A very recent study used BA for the detection of HIV-1 capsid (p24) antigen, an important marker to detect the decreased $CD4^+$ T-cell decreased count, enabling a timid, HIV-1 infection detection. The screening method used the anti-p24-coated microplate for the early detainment of the p24 coat, followed in line by the biotinylated detector Ab. Subsequently, the streptavidin-functionalized NPs-based BA DNA was optimized for likely signal amplification. This is followed by the detection using a chip-based scientometric method. The optimized probe displayed a (0.1–500) pg•ml^{-1} LOD, with ~150-fold higher sensitivity compared to conventional ELISA. The commercial feasibility was screened

using clinical blood samples, with 100% negative and positive predictive outcomes in 30 and 45 samples, respectively. Apart from this, the HIV-1 detection consumed 3 days lesser compared to the ELISA for seroconverted samples.

- **Metal nanoparticles**: These entities are also efficient signal amplification labels for biorecognition sensitivity enhancement, imbibed by their quantum mechanically distinguished optical attributes. Several studies have explored Au and Ag NPs as they exhibit the plasmon absorbance bands in the visible light spectrum and are determined in turn by the size of their respective particles. Thereby, the spectral variations arising out of the aggregation and monodispersed states have been the basis of biomaterial-metal NPs-integrated functional systems as the detection amplifiers. One study has, for example, used Au-labeled antibodies as an integrated part of a microfluidics chip for simultaneous diagnosis of HIV and syphilis from 1 μl of whole blood. The reduction of Ag^{+} on the Au NPs comprises signal amplification within a millimeter-sized channel provision. Thereafter, the OD of silver film is detected and is quantified using low-cost optics or qualitatively through the naked eye. A ccomparative analysis of this sensing configuration with commercial ELISA kits revealed a 100% sensitivity and (98–100)% specificity for HIV and (82–100)% sensitivity, (97–100)% specificity for syphilis.
- **Magnetic nanoparticles**: The signaling amplification using MNPs-coupled detectors in biosensors offers the advantage of compatible utility in solution-phase sandwich assays like that of diagnostic magnetic resonance. The distinction between solution-phase assays is the relatively faster assay times compared to diffusion-dependent surface structure-based assays. The diagnostic magnetic resonance exhibited by MNPs is described by the presence of capture as well as detection moieties in solution, wherein both are linked to MNPs. On screening a potential analyte of interest, magnetic particles cluster as antibodies and bind the analyte. The clusters of magnetic particles are more efficient at dephasing nuclear spins of adjacent water protons, decreasing the spin–spin relaxation time and generating a quantifiable signal. In a significant attempt, Chung and colleagues developed a magneto-DNA platform targeting bacterial 16S rRNAs, capable of profiling a panel of 13 bacterial species from clinical samples as diverse as urine, pleural fluid, biliary fluid, ascetic fluid, and blood. Concomitantly, reverse transcription-PCR amplification of 16S rRNA, polymeric bead capture conferred target DNA enrichment, and lastly, magnetic amplification via conjugated magnetically labeled beads to the target DNA can collectively enable a single bacterium DS. A single MNP is capable of disturbing the native chemical balance of billions of water molecules residing in the vicinity. Potential drawbacks of the magnetic bead DS comprise the manual sample preparation along with PCR analysis as a separate step from the nuclear magnetic resonance-based sensor.

5.1.1 Label-Free Assays in Biological Sensing

These assays of a biosensor functioning monitor the changes that occur when target analytes bind with molecular-capturing elements immobilized on a solid support or exhibit variations in the interfacial capacities or resistance. Such assays require merely a single recognition element, paving the way for a simplified assay

Table 11 The whereabouts of label-free sensing mechanisms for infectious diseases, underlining the working principle, distinctive prospects, and the lacking aspects

Assay configuration	Technology	Distinctions	Uncertainties
Optical transducer	Surface plasmon resonance	Real-time detection and high-throughput possibility	Diligent to sample matrix changes, cumbersome sensor surface functionalization, and bulky optical probe
Electrical transducer	Redox electrochemistry (amperometric) Impedance spectroscopy Potentiometry Field-effect transistor	Simple sensor design, robust detection platform: flexible for miniaturization Simple electrode design, real-time detection Real-time detection and possibility of consecutive measurements on different samples Real-time detection, stable sensing response, and POC-compatible detection platform	Requires redox species to increase current generation, no real-time detection, and vulnerable to sample matrix effects Sensitive to sample matrix effects, bulky equipment, and no trivial data analysis Bulky equipment, sensitive to sample matrix effects, cumbersome sample preparation, and mandates temperature control Vulnerable to sample matrix effects, complicated sensor configuration, and acute dependence on temperature control
Mechanical transducer	Microcantilever Quartz-crystal microbalance	Real-time detection, multiplex, and high-throughput possibility Simple electrode design, real-time detection, and detection platform compatible with POC configuration	Vulnerable to sample matrix effects, mandates diligent temperature control, and bulky equipment Vulnerable to sample matrix effects, diligent temperature control, and stress adjustment

configuration, and reduced detection time and reagent cost. This mode of recognition is particularly useful for small molecular targets that can be buried within the binding pocket of capturing element. This leaves a very low chance for a possible interaction with a detector agent that is required for a labeled assay. Yet another advantage of the label-free detection method of biosensing is the steadfast ability to quantitatively assess the molecular interactions in real time, accompanied by continuous data recording. Furthermore, the target agents can be detected in their natural form (without labeling and chemical modification) and can be preserved for further analysis. Label-free sensing for infectious diseases is made feasible through a binding event-generated distinction in optical, electrical, or mechanical signaling (Table 11). Readers are requested to see the details of referred studies in this section in the Sin et al. 2014 literature source as we are subject to the restriction of not including more than 50 references.

- **Optical transducers**: The energy converters of optical sensors are characterized by their precise involvement in optical phenomena such as changes in surface plasmons and interferometry. As also elaborated previously, surface effect in the form of SPR is the excitation of an electromagnetic wave propagating along the interfering direction of two interacting media. It is mandatory herewith that the interacting media should have dielectric constants of different signs, like those in a metal and sample buffer prepared using a specific angle of the incident light beam (Sin et al. 2014). The output signal is generated via total internal reflection (TIR), resulting in a reduced intensity of reflected light. The inclination at which resonance happens is minutely affected by the variations in refractive index, corresponding to the nanoscale-thick film formation on account of surface molecular interactions. The variations could be screened by analyzing the least shift in light intensity over a defined time lapse. A useful attempt herein used SPR-driven biosensor for the detection of *Escherichia coli* (O157:H7) and methicillin-unresponsive *Staphylococcus aureus* using T4 and B14 bacteriophages as detaining organisms. This biosensor could screen 1000 cfu•ml^{-1} within 20 min duration.
- Another strategy for bettering the functional accuracy of optical biosensing is backscattering interferometry (BI), working through a singular monochromatic light source (low-power He–Ne or red diode laser), optimized for focusing over a microfluidic channel. The reflected light intensity is monitored via a detector. The typical operation involves a coherent illumination of a fluid-filled channel, from the light source, generating varied interferences due to the sub-wavelength structures of the channel. Processing of output response is made via analysis of fringe pattern using a detector positioned in the backscattered direction through which changes in refractive indices could be monitored to quantify the underlying molecular binding patterns. The working configuration is optimized for screening via free solution or surface-immobilized molecular proximities, which are generally discrete for microfluidic devices. Detection to the picoliters extent is illustrated via determining the binding constants ranging from micro- to picomoles. A select study (Kussrow & colleagues) herein demonstrated a rapid screening of human IgG from syphilis sufferers, making use of a purified recombinant treponemal antigen r17, explaining the significance for serological diagnosis in a clinical specimen.
- The label-free configurations of optical biosensors usually necessitate an accurate placement of light for coupling with the sensing region, a critical aspect for point-of-care applications. Thereby, optical sensing can be significantly advanced using the BI modulation summed up over the different sample regions. Integrated optical analysis facilitated the positioning of multiple active and passive optical components over the same substrate, allowing the configuration of multiple sensors over a single chip. A recent attempt herein comprised the design of a nanobiosensor working via light transmission in plasmonic nanoscale perforations and explicit classification of specific antibodies for multiple diverse strains of rapidly developing viruses. The optimized configuration involved a direct coupling of perpendicularly incident light using sensing assays that eased the

requirements for photocoupling. Analysis of the performance on a clinical scale was estimated via detection of single-enveloped RNA viruses (vesicular stomatitis and pseudotyped Ebola strains) and squarely the largely masked DNA viruses.

5.1.2 Electrically Stimulated Transducers

Majorly attributed to their high sensitivity, simplicity of application, and the ability to be coupled with automated, miniaturized processing tools, the sensing mechanisms using electrical and electronic signal modulation are reasonably well demonstrated. To date, the most reported working principles of electrical biosensors for the diagnosis of infectious diseases comprise voltammetric, amperometric, impedance, and potential difference analysis. Both voltammetric and amperometric sensors function via measuring the electrical current passing through an electrolyte in terms of direct current potential difference between the two electrodes in an electrolyte, with time.

- A noted attempt in this regard is the study by Qiu and colleagues, wherein the investigators developed an immunosensor for amperometric detection of hepatitis B surface antigen. The working configuration of the probe involved a glassy carbon electrode modified with a network of cationic poly(allylamine)-fragmented ferrocene and anionic Au NPs. This combination enabled the formation of a biocompatible, stable thin film having a high specific interfacial area that minimized the loss of mediator as well as antibodies through accurate and selective adsorption to hepatitis B surface antigen.
- Electrical biosensors working through monitoring impedance changes function by resisting the current flow on the application of a sinusoidal potential difference over a wide range of frequencies, with a constant DC bias potential difference. Impedance is computed as the ratio of applied sinusoidal potential differences (generally varies) and the concomitant current output generated across the interface.
- Multiple studies screened infectious disorders using well-characterized impedance-driven biosensors. In one of these noteworthy attempts, α-mannose carbohydrate was recognized to screen the presence of *E. coli* ORN 178, a surrogate for pathogenic strain O157:H7. The optimized configuration exhibited an LOD of 10^{-2} CFU (colony-forming units) per ml as its LOD. Another rigorous study by *Shafiee and accomplices* screened enriched HIV-1 and its multiple subtypes having magnetic beads functionalized with anti-Gp120 (HIV core protein) antibodies. Viral lysates were screened using impedance measurement to the ultra-low extents of (10–6 to 10–8) units at ml extents on an electrode having simplistic geometry. Both of these studies pinpointed discrete and specific recognition of a small molecular target that was possible due to nearly null interaction with the detecting probe, a major feature of labeled assay configuration. A prominent need highlighted in both cases, however, mandates that capturing elements should be capable of accommodating the analyte within a restricted binding domain.

- Another method for the detection of analytes using the potential difference measurement based on the measurement of accumulated charge using a high-impedance voltmeter with the trifling current flow is "potentiometry." A significant attempt herein demonstrated an immunosensor that worked through the potentiometric transduction ability of single-walled CNTs in combination with protein-explicit RNA aptamers. The net aim centered on the identification of variable surface glycoproteins (VSGs) of African Trypanosomes. Aptamers are fundamentally the small and synthetically made RNA/DNA segments that are capable of forming secondary or tertiary structures for binding to specific molecular targets. A major limitation curtailing the functioning of such assays is the short half-life of RNA aptamers, characterized by the receptiveness of phosphodiester backbone alongside the ribonuclease and exonuclease-bound 5′ and 3′ termini. Keeping this in mind, the study focused on the synthesis of nuclease-resistant RNA aptamer sensors having 2' F substituted cysteine and uracil nucleotides. To summarize, the functional sensing domain (VSG-specific, nuclease-resistant RNA aptamers hybridized with SWCNTs) worked well for attomolar extent detection of VSG protein in the blood.
- A fairly similar sensing mechanism involves FET, making use of a moderate current passage of a semiconductor via an externally applied electric field due to the charged particles in the vicinity. In general, the response of this sensing mechanism is accomplished in terms of threshold potential shift of field-effect structure, owing to the binding at the gate electrode. The optimized sensing assay was used for the detection of the pathogenic yeast strain, *C. albicans*, through a network of SWCNTs functionalized with monoclonal anti-*Candida* antibodies, integrated functioning as a conductor channel. The specific detection of yeast membrane antigens was conceptualized through this sensor at an LOD of 50 cfu•ml^{-1}.
- The explicit probes working through assessing the FET dynamics exhibited stability over a wide pH range and electrolyte concentrations, besides being generated in large concentrations at a relatively low cost. These accurate sensitivities for the nucleocapsid protein detection by the FET platform comprising biosensor, in a complex media, equated the functioning with an ELISA protocol.
- **Mechanical transducers**: Recent advances in microelectromechanical systems (MEMS) and nanoelectromechanical systems (NEMS) have added a new realm in the working sensitivity of transducers capable of perceiving physical stimulus changes such as those in forces, motions, mechanical properties, and mass accompanied by molecular recognition. Among the different mechanical biosensors, cantilever and QCM comprise one of the most accurate probes. Mechanical bending of micro- or nanocantilever is screened through an optical readout mechanism optimized for accurate detection of stress/strain profile of the cantilever promptly after an analyte binds the probe. A notable attempt by Mader and colleagues herein functionalized a cantilever array with carbohydrate molecules to capture *E. coli*. The probe used by investigators involved Au coating of the top edges of the cantilever array functionalized with self-assembled layers of distinct mannosidase. The assembly facilitated a real-time reproducible detection of

diverse *E. coli* strains including ORN 208, 178, and 206, with a sensitivity ranging well above four orders. Structural similarity of galactose with mannose paved the way for accommodating an internal reference cantilever with galactose to screen the nonspecific binding and concurrent nonspecific reactions, such as changes in pH, refractive index, or the reactions occurring on the cantilever underside.

- In a closely related study, Liu and colleagues used cantilever-driven biosensing to ascertain real-time cellular growth via monitoring the drug–cell interactions. The detected stimulus comprised the real-time growth of *S. cerevisiae* strains, YN94-1 and YN94-19, using the polymer cantilevers. The static mode of cantilever scanning exhibited an enhanced sensitivity, distinguishing the impacts of energizing the interactions between essential nutrients (completely synthetic uracil) and 5′-fluoroorotic acid in the yeast cells. In place of preferred silicon nitride cantilever probes, polymer-based probes were designed at a moderate expenditure with much lesser micromachining, encompassing a high sensitivity by tough polyimide modulus.
- The detection using piezoelectric mechanisms, involving QCM, works through assessing the dynamics of resonant frequency for oscillating quartz-crystal, in response to the alterations of surface-adsorbed mass, following the biorecognition. The discrete analyte identification was based on the internal mechanical stress generated in a piezoelectric responsive substrate, being coupled with an external potential. This stress gives rise to a varying electric field that in turn generates an acoustic wave throughout a crystal in a perpendicular orientation concerning the plate surfaces. A change in the QCM resonance frequency depends on multiple aspects, among which changes in mass, viscosity, the dielectric constant of the solution, and ionic status of the crystal–buffer solution interface are prominent. A noteworthy effort herein from Hewa and accomplices reports a QCM-based immunosensor for screening influenza A and B viral strains via conjugating Au NPs to anti-influenza A or B MAbs. The optimized configuration exhibited an LOD of 10^3 pfu•ml^{-1} for laboratory culture preparations and clinical-grade analysis (nasal cleansers). Screening for clinical grade revealed a significant comparison of as-prepared biosensor with standard shell vial and cell culture assays, analyzed for 67 clinical specimens, overall possessing higher sensitivity and specificity than ELISA.
- Still another way to enhance the sensitivity and specificity of QCM biosensors involved the imprinting of a molecular-grade film over a QCm chip. Polymers imprinted molecularly have emerged as vital accessories in the fabrication of synthetic recognition elements. This is very well illustrated by the work of Lu and group, who demonstrated a biomimetic sensor generation via epitope imprinting for the detection of HIV-1 glycoprotein, Gp41. This glycoprotein has been demonstrated as an explicit marker for HIV infection progression and the therapeutic response *vis-a-vis* administered therapies (antibodies). Better affinity-minimized random (nonspecific) interactions and cost-effectiveness are the major benefits of epitope-driven imprinting over the traditional approach involving proteins. In another significant attempt, dopamine was employed as a

functional monomer for the Gp41 detection, comprising polymerization on the QCM chip surface in the presence of a synthetic peptide analogous to 579–613 residues of Gp41. The optimized configuration enabled quantitative Gp41 sensing with 2 ng•ml^{-1} LOD, an extent comparable with that of ELISA. The sensor also functioned well in the screening of Gp41 spiked human urine samples, exhibiting feasibility for point-of-care use.

- Another closely matching probe was reported by Tokonami and colleagues, wherein a molecularly imprinted polymer film comprising excessively oxidized polypyrrole (OPPy) in combination with QCM was used for direct bacterial detection. The optimized setup enabled an LOD of 10^{-3} CFU per ml or lesser extents within 3 min. Bacterial cavities created in OPPy film exhibited a high selectivity, enabling the distinctive recognition of the target bacterium, *Pseudomonas aeruginosa*, in a mixture containing uniformly similar morphology bacteria, comprising *Acinetobacter calcoaceticus*, *E. coli*, and *Serratia marcescens*.

As label-free sensing configurations generally do not rely on signal amplification, enhancing the detection specificity and sensitivity is majorly accomplished via an optimum selection of sensing probes and transducers. Recent advances in bioanalytical methods and characterization assays are highly aimed to design and implement improved sensing probes having significantly high sensitivity, specificity, and long-term stability. A formidable challenge limiting the clinical utility of label-free biosensors is the replication of the identification principles from laboratory-scale to commercial-grade clinical samples, such as blood, serum, and urine. This is substantially attributed to the complex matrices of clinical samples, which may result in nonspecific, random binding and generation of aberrant signals. For instance, charge-driven label-free biosensors are highly affected by even minute changes in pH, ionic strength, and vicinity temperature. Likewise, nanowires generally mandate sample desalination before the detection while microcantilevers need precise temperature regulators. The random binding-driven interactions are the common causes of loose control, leading to a measurable signal that is not much distinct from the one generated by the targeted analyte. Several alternative methodologies are documented to moderate the matrix interference of samples. The most common of these involves the design of hydrophilic antifouling surfaces such as PEG, whose surface modification is reported as a befitting biomarker for screening the clinically significant sensitivity in the native blood serum using EIS. Another strategy uses zwitterionic polymers with a hydrophilic receptiveness and electrically neutral character, being of significant use as antifouling interfaces. It is important to recall here the observation of multiple investigations, wherein coating the probe surface with poly carboxy betaine methacrylate (a zwitterionic polymer) inhibits the random and nonspecific protein adsorption from the blood serum. This significantly improves the antibody–target-binding affinity, making it possible to screen clinical samples through a label-free detection (Sin et al. 2014).

6 Case Studies on Biosensor-Diagnosed Cancers

Cancers are perhaps one of the worst mortalities affecting mankind at present. Even though consistent progress in molecular characterization and diagnostic assays has collectively improvised their pathological know-how, timely treatment is badly recognized as the need of the hour. Irrespective of the organ concerned, hereditary factors and mutational onsets are the major manifestation factors of all cancers. Of late, the treatment methodologies have though bettered but only in terms of curative aspects and not much with a preventive outlook. Persistent trouble with the efficient treatment of cancers pertains to their metastasis and changing mutational consequences in the aftermath of environmental variations. For instance, lung cancer (LC) has a particular higher probability to affect smokers, whereby more rigorous information on stage-specific biomarkers is inferred. Although such events are less observed in nonsmokers, manifold reasons, including genetic factors and a higher risk of passive smoking, may accumulate viciously. Similarly, the geographical specifications affected by unprecedented mining activities are more vulnerable to deteriorating soil quality (deposition of heavy metals and radioactive elements), wherein the chances of ophthalmological and throat tumors are aggravated. Likewise, Asian and African females are more susceptible to breast cancers (BCs) than European females. For the treatment of cancers, early diagnosis is a must so that the spread to nonoriginating locations is minimized. To ensure this, it is highly urgent to screen the initial stage biomarkers through which the efficacy of treatment protocols could be modified as per the severity. Since this chapter is not attributed to cancers and their molecular aspects, a description of the emerging biosensor-mediated fastened treatment of more threatening, LC and BC is described in detail with others being briefly summarized.

6.1 Lung Cancer

- Prevailing as a molecular heterogeneous disorder, LC mandates a need for robust and accurate therapy for adjudging its pathological manifestation. On a histological basis, LC prevails as small-cell (SCLC) and non-small cell (NSCLC) subtypes, with ~85% of sufferers being affected by NSCLC. Notable NSCLC subtypes include lung adenocarcinoma (LAD) and lung squamous cell carcinoma (LSC). The latest research analysis predicts tobacco smoking as the leading LC cause, contributing to >80% of the global cases. Despite all forms of LC exhibiting some sort of association with tobacco smoking, the LSC and SCLC are more dependent on smoking than LAD. LAD affects nonsmokers more commonly, facing a higher vulnerability of having LC from sources other than tobacco smoke (Hackshaw et al. 1997).
- However, despite smoking being a major cause of increasing LC mortality, present treatment options are the outcomes of a paradigm shift from generalized cytotoxic therapy to personalized medicine. Indeed, treatments do assume a specific regime in light of swift genetic modifications and varying programmed

Table 12 Overexpressed cell surface receptor proteins of lung cancer cells

Sr. no.	Receptor noticed in specified LC cell line	Associated cellular function and possible clinical application
1.	EGFR studied in A549 LC cells	Cell surface tyrosine kinase protein receptor involved in growth, division, and proliferation, and EGFR targeting chitosan NP silenced the mitotic checkpoint, Mad2, and induced cell death
2.	CD44, acutely expressed in NSCLC tumors and hyaluronic acid, noticed in A549 and SK-LU-1 LC cells	Targeted for (30–60)% tumor growth inhibition, promotes macrophage repolarization and activates p53-mediated apoptosis
3.	Transferrin, type II transmembrane glycoprotein involved in cellular Fe transport and growth regulation, noted in H460 human LC cells, and expressed in 76% LAD and 96% SCLC cases	Low use and limited therapeutic success, but acts significantly in the blood–brain barrier (BBB)
4.	Folate receptor alpha (FRA)	74% LADC and 13% SCLC overexpress FRA, targeted via polyspermine NPs conjugated with folic acid, FA-conjugated chitosan graft PEI-mediated small hairpin delivery to cancer cells, induced stable shRNA condensation, and DNAse protection
5.	Integrins noted in H1299 cells	α5, β1, and αvβ3 are common in all LCs, and αvβ3 is used for improved diagnosis involving PEG-grafted nanoparticle surfaces Induces EGFP gene silencing in H1299 cells, and PLGA NP promoted VEGF inhibition in a mouse model

Note: These entities are the crucial riders of implicit identification of tumor cells and their characteristic expression is used as a diagnostic measure for tumor cells

death ligand-1 (PD-L1), the latter being vitally involved in escaping the targeted therapies through immune checkpoint blockers (ICBs). Table 12 pinpoints the overexpressed cell surface receptor proteins in LC, giving rise to significant intra-tumoral heterogeneity with distinct molecular characteristics. These proteins are authentic biomarkers of LC manifestation, with Kirsten rat sarcoma (KRAS) and epidermal growth factor receptor (EGFR) being the genes undergoing rapid mutations, noticed with greater frequency in LAD and LSC. Several serum tumor markers have been studied in LC, including carcinoembryonic antigen (CEA), CA-125, CYFR A21-21, chromogranin A, neuron-specific enolase (NSE), retinol-binding protein (RBP), α1-antitrypsin, and LSC carcinoma antigen.

- No single blood test is known for LC, and CEA is a widely studied tumor marker, reported as elevated in (0–38)% of SCLC patients and (30–65)% of those having the extensive disease. Studies estimate elevated CEA in (30–65)% of NSCLC

sufferers, with a retrospective investigation of 153 NSCLC patients (entirely resected tumors) by Muley and colleagues reported lower overall survival rates for elevated CEA expressions contrary to those of normal. Both CEA and CA-125 are expressed to a lower extent in patients having the early-stage disease compared to that with metastatic manifestation. Aggressive pathology, coupled with an advanced stage at diagnosis and a high relapse propensity, is the major cause for a below-par SCLC prognosis to date. The data regarding the identification of potential biomarkers against LC are substantially retrospective with a dearth of larger prospective trials. A significant role of post-translational modifications has made proteomics an emerging platform (superseding genomics) to screen the corresponding effect on carcinogenesis.

- To date, however, 2D-gel electrophoresis is extensively used to screen the potential SCLC and NSCLC biomarkers, but the overall process is cumbersome with low sensitivity. The limited extent of available tissue is another factor affecting the identification of potential LC. Thereby, screening the optimum serum biomarkers could be of much clinical significance, so plasma proteome analysis is a complex undertaking, having witnessed significant advances. Using multidimensional chromatography and mass spectrometry (MS) analysis, as many as 1175 LC implicit plasma proteins have been identified. Besides electrophoresis and MS, other notable methods for LC detection developed in the last decade include computed tomography (CT), chest radiograph (CRG), magnetic resonance imaging (MRI), positron emission tomography (PET), and biopsy. Quite disappointedly, all these methods suffer from poor sensitivity besides being expensive and inducing physical/chemical risks. As a result, they could not enable early-stage LC diagnosis. Although subsequent methodologies of ELISA and PCR did exhibit high sensitivity besides having less invasive procedures, the detection progress is slow, along with an expensive consumption of reagents.
- Of late, several robust mechanisms have emerged to steadfast the LC diagnosis using the fluid mechanics dynamics; visible as electrochemical, optical, microfluidics, and microarray-based biosensors (refer Yang et al. 2019 for details). Compared to the conventional methods, the novel sensing mechanisms in these biosensors have better sensitivity, selectivity, and stability besides being a moderate cost and relatively simpler operational modes. A common factor in the emerging progress of these biosensors has been the emergence of multifunctional and size-varied energy possessing NMs. The functional diversity of NMs manifested from their high SA, multiple interactions at the same instant, and size-driven energy modulation is the reason for improved detection limits and reduced detection times in these biosensors. For accurate screening of LC, it is pertinent to have a sound knowledge of various early-stage LC biomarkers that are described next.
- In most general words, markers are the biochemical parameters that can be measured in the plasma or other body fluids isolated from suspected cancer patients, commonly exhibiting a susceptibility of being the prerequisite for serological tumor marker diagnosis. The typical expression of tumor markers at

a given instant is determined by several tumor parameters such as size, mass, expression extent, synthesis ability, and catabolic and excretion rates besides the tumor blood supply. Variation in any of these factors is likely to aggravate the complexity, leading to enhanced sensitivity of tumor marker detection. In the last decade, DNA methylation has emerged as a prominent sensitive and specific epigenetic event that has been reported for accurate screening of LC pathological changes. It is significant to mention here that the expression of the abovementioned LC markers may have an association with smoking history, ethnicity, and genetic factors but such dependence is merely quantitative. So, the desired need is to have accurate and sensitive molecular characterization tools that can screen even a minute expression of these. The following points shed light on some prominent markers among these. For cited literature in the subsequent subsections, readers are suggested to refer a 2019 review article by Yang and associates, featured in the *Journal of Biosensors and Bioelectronics*.

- **Neuron-specific enolase**: With a 39 kDa molecular weight, neuron-specific enolase (NSA) comprises two matching polypeptide chains (α/γ or γ/γ). NSE is essentially glycolytic enzyme enolase that substantially prevails in peripheral neurons besides central and neuroendocrine tissues. The occurrence of NSE in SCLC was reported first in the 1980s, with all sufferers showing a high NSE expression. NSE release may occur in erythrocytes and blood platelets with a concentration variation that is independent of age, sex, and smoking history. In 2003, it was reported that NSE also served as an important NSCLC marker, being recognized as a survival indicator irrespective of erstwhile prognostic factors.
- **Cytokeratin 19 fragment**: A 36 kD cytokeratin 19 fragment, **cytokeratin 19 fragment** (CYFRA 21-1) reside in the epithelial cell (including bronchus epithelium) cytoskeleton and is the only source of CYFRA 21-1. CYFRA 21-1 has been screened as exclusively expressed in lung tissues and is the most sensitive NSCLC marker (exclusively in LSC). Studies based on multivariate analyses unanimously reveal CYFRA 21-1 as an important marker for screening the NSCLC prognosis, treatment efficacy, and recurrence.
- **Carcinoembryonic antigen**: With a molecular weight of ~180 kDa, carcinoembryonic antigen (CEA) is a cell-membrane-associated glycoprotein group, expressing to the highest extent in the fetus serum of 22nd pregnancy week (not in a healthy adult). The importance of CEA as a tumor biomarker is not limited to LC, and it is also a keen identifier in the colon, gastric, pancreatic, breast, and ovarian cancers. The normal CEA range for adult nonsmokers and smokers is <2.5 ng•ml^{-1} and 5 ng•ml^{-1}, respectively. A rise above these concentrations signifies the recurrence or progression of LC. A high CEA concentration in the exhaled air condensate is perhaps the best predictive aspect of early NSCLC. The screening of lung malignancies is usually made via combined detection of CEA and CYFRA (a 21-1 extent).
- **Squamous cell carcinoma antigen**: This is a 48 kDa protein, associated with the serine protease inhibitor family. The squamous cell carcinoma antigen (SCCA) prevails in squamous cells and is referred to as a structural protein, indicating a differentiation cancer stage. Variations in SCCA extents are the familiar

indicators of lung squamous carcinomas, cervix, uteri, head and neck regions, and esophagus. Varied SCCA expression levels inferred different LC stages; an NSCLC monitoring by SCCA mandates its screening in combination with CYFRA21-1.

- **Carcinoma antigen 125 (CA125)**: This is a 200 kDa membrane mucin-like glycoprotein and prevails as a prominent BC, ovarian cancer, and LC biomarker. Among the different LC forms, adenocarcinoma and large cell LC exhibit significantly high carcinoma antigen 125 expressions. Thus, CA125 can be used as a predictive marker for evaluating the prognosis, treatment accuracy, and early treatment response in NSCLC.
- **Tissue polypeptide antigen (TPA)**: This is a cytoskeleton protein having 20 kDa, first noticed as being released from proliferating cells originating from ER and cell membrane. TPA can independently exhibit a significant LC prognosis.
- **Others**: Besides the abovementioned LC markers, some other markers used to screen the LC include carbohydrate antigen 19-9, tumor M2-pyruvate kinase (M2-PK), progastrin-releasing peptide (ProGRP), vascular endothelial growth factor (VEGF), serum human epididymis protein-4 (HE4), cancer-testis antigen (NY-ESO-1), clinical application markers such as CRP and lactate dehydrogenase (LDH). Besides these, a range of newly discovered markers for LC includes exosomal microRNA, navitoclax, TFIIB-related factor 2, DR-70, Glasgow prognostic source, serum microRNA 21, and serum-microRNA 204, serum microRNA-100, apurinic/apyrimidinic endonuclease 1, and urokinase plasminogen activator.
- Erstwhile of the above serum markers, those belonging to bronchoalveolar lavage fluid (e.g., ubiquitin-specific peptidase 8, chitinase 3-like 1, glutathione-s-transferase P1) and breath markers (e.g., 2,4-dimethylheptane, 2-methyl-1-pentene and 4-methyloctane) are also the prominent LC markers.

6.1.1 Biosensors for Lung Tumor Biomarker Detection

Updates corresponding to this section are almost summarized from the 2019 review article by Yang and associates, featured in the *Journal of Biosensors and Bioelectronics*. Thereby, readers are suggested to refer to the details of non-listed references from this literature source.

1. **Neuron-specific enolase detection**: Possessing a significant specificity toward NSCLC, the NSE screening has been made using electrochemical, optical, and surface-enhanced Raman scattering (SERS)-driven biosensors. A high sensitivity, rapidity, and simplicity of operation are responsible for a wider application of electrochemical and optical analytical methods. Several NMs and biocompatible polymers are being applied to design biosensors to achieve stable and greater signal amplification. The following sections describe the recently reported NSE sensing configurations using electrochemical, optical, and feasible NMs.

6.1.2 Electrochemical Biosensors

The biosensors working on an electrochemical basis for NSE detection can be labeled or label-free in configurations and may use NMs or polymers for functioning through a sensitive platform. The labeled configurations usually amplify the detection signal as per the modified platform and labeled tag. Contrary to this, the label-free version merely requires a modified platform for signal amplification. In a 2017 attempt, Wei and his team configured the electrochemical biosensors for NSE detection using Au NPs and reduced graphene oxide composites-driven signal intensification. The Au NPs-reduced GO enabled the enhanced binding sites for modified Ab, thereby enhancing the detection signal sensitivity. The designed sensor configuration exhibited a linear relationship from (0.1 to 2000) ng•ml^{-1} with 0.05 ng•ml^{-1} LOD. To further enhance the DS, the investigators used porous 3D graphene–starch architecture as a sensing platform with Au NPs-coated ordered mesoporous carbon–silica (OMCSi–Au) as a target. The introduced modifications enabled a higher surface area to capture the target proteins alongside accelerated electron transfer, resulting in a 0.008 pg•ml^{-1} LOD.

The simplified configurations of the label-free assay have been the reason for their greater use in NSE detection with comparatively higher stability. In a 2018 attempt, Zhang and his team used a 3D macroporous reduced GO-polyaniline film-engineered Au electrode for NSE detection. The rGO-PANI composite exhibited a larger specific surface and high electron conductivity with manifold electroactive sites. To overcome the vulnerable stability of sensing interactions, the molecular imprint (MIP) served as artificial receptors for implicit target molecule recognition. Another configuration was reported by Wang and colleagues, who used a 3D structured Au nanoarray as amplification moiety and 1-(3-mercaptopropyl)-3-vinyl-imidazolium tetrafluoroborate as the molecularly imprinted film, on the removal of NSE templates. The NSE detection response exhibited a linear range from (0.01 to 1) ng•ml^{-1} with an LOD of 2.6 pg•ml^{-1} and $<5\%$ relative standard deviations (RSDs). Another study aimed at NSE detection in serum samples used a wireless POC testing system constituted of Au NPs nanocomposites, thionine, and amino-functional graphene as a microfluidic paper-based analytical device and a smartphone as a signal receiver for NSE detection. The sensor exhibited a low LOD along with a wide detection assortment in serum samples that argued well for clinical applications.

6.1.3 Optical Biosensors

As opposed to electrochemical mechanisms, the optical biosensors exhibit a low interference with the detection system, commonly used to detect biological targets. Amalgamating electrochemical and optical detection mechanisms, the photo-electrochemical (PEC) analytical methods are well compatible with a more sensitive NSE detection. To elucidate this, Lin and colleagues designed an enzyme-free multicolor immunosensor for NSE detection. The sensor utilized Cu^{+2}-modified carbon nitride nanosheets as catalytic substrate with Au nanobiopyramid as a chromogenic substrate for multicolor display when TMB^{2+} etches Au nanobiopyramid and develops multicolor patterns.

6.1.4 Other Biosensor Configurations

The SERS has been viciously reported for immunosensing purposes, with a modifiable surface for biomolecule conjugation. A potential attempt in this regard by Gao and colleagues prepared nanostar Au morphology that was then conjugated with malachite green isothiocyanate (MGI) after being functionalized with silica NPs (Si NPs). The assembly was used as SERS probes via applying disposable paper-based lateral flow strip as a platform and Au nanostars coupled with MGI-Si NPs as detection probes. The biosensor exhibited a wide detection range of (1–50,000) ng•ml^{-1} and 0.86 ng•ml^{-1} LOD in diluted blood plasma samples.

2. **Cytokeratin 19 fragment 21-1 detection**: Recognized as the most effective NSCLC biomarker, CYFRA21-1 exhibits high sensitivity and specificity. Disappointingly, very few studies are reported on CYFRA21-1 detection, and the necessary aspects of electrochemical and optical biosensors are discussed next.

6.1.5 Electrochemical Biosensors

The labeled configurations are rather more common for CYFRA21-1 detection. In one such attempt, Zeng and colleagues developed a labeled biosensor using 3D graphene oxide, chitosan, and glutaraldehyde as platform and Ab-modified nanocomposites (prepared using –NH_2 functionalization of MWCNTs), thionine, Au NPs, and HRP-labeled Abs as a label. Another attempt by Gao and associates reported an immunosensor using Si nanowire tunneling FET (optimized by CMOS compatibility). The bottom-up approach of tunneling FET immunoassay can detect CYFRA21-1 extent as low as 0.65 fg•ml^{-1}. Other than frequently used NMs, studies have used rare earth metal hydroxides and hydrogel assemblies for CYFRA21-1 detection. A study by Tiwari and colleagues has shown that lanthanum hydroxide ($La(OH)_3$) NPs were immobilized on an indium-tin-oxide glass substrate as a signal amplification platform due to a larger number of bioactive sites, high electron transfer mobility, and electrocatalytic response. The developed immunosensor exhibited high sensitivity for CYFRA21-1 detection with 5 min response time. Another study by Wang and Ma reported enhanced DS of amperometric biosensors using a conductive hydrogel. The probe involved functionalized cross-linked phytic acid (containing Au NPs) for CYFRA21-1 detection, resulting in unmatchable redox activity and electron transfer ability, developing a linear response within (50 fg•ml^{-1} to 100 ng•ml^{-1}, with an LOD of 38 fg•ml^{-1}). Readers are requested to find the details of the above-referred studies in the 2019 *Journal of Biosensors and Bioelectronics* contribution by Yang and associates.

6.1.6 Optical Biosensors

Low interference, weak background nature, and high sensitivity are the features of an optical immunoassay for CYFRA21-1 detection. For instance, Yu and colleagues, through their 2019 research attempt, developed a novel PEC biosensor having biofunctional polydopamine/tungsten oxide NPs as a sensing platform. Besides these, the SPR-based biosensors relied on light-stimulated electronic oscillations at the changeable metal film surface, which can be as such used for CYFRA21-1

detection. Another advantage of SPR biosensors is their high stability and sensitivity for plasma screening. As a paradigm shift, Chiu and colleagues in 2018 fabricated an ultrasensitive SPR immunosensor based on cystamine as a linker on a GO sheet immobilized on the chip surface. The biosensor exhibited significant stability and sensitivity and revealed kinetically fitted values in the human plasma. Owing to legal restrictions, the referred studies could be traced to the 2019 *Journal of Biosensors and Bioelectronics* contribution of Yang and associates.

3. **Carcinoembryonic antigen detection**: Noted as a broad-spectrum tumor marker, screening the CEA expression is viciously recognized as an implicit signature for diagnosis, prognosis, and monitoring the different cancers. Compared to other biomarkers, a higher molecular weight of CEA enables a larger number of functional groups for immobilization and modification. This has been the reason for a range of biosensors being available for CEA detection, including electrochemical, optical, SPR based, SERS-based, aptasensors, and others. The following sections discuss the recent progress in biosensors design for CEA detection.

6.1.7 Electrochemical Biosensors

The recent surge in electrochemical biosensor development for CEA detection is substantially due to their fast response, stability, high sensitivity, and specificity. Apart from this, the attainment of high signal amplification has been made possible by the incorporation of different NMs, including noble metal NMs, carbon NMs, and polymeric NMs. Among the electrochemical options, much interest has been in labeled configurations, wherein Lee and colleagues 2017 designed a sandwich biosensor using Ag NPs in combination with rGO (AgNPs-rGO) for modifying the SPCE as sensing matrix and HRP-labeled Ab as a tag for CEA detection. The sensor exhibited a (0.05–0.50) $\mu g \bullet ml^{-1}$ calibration with 35 $ng \bullet ml^{-1}$ as LOD. The preferential benefits of using NMs in place of enzymes comprise remarkable stability, better sensitivity, and improved modification. As per Feng and colleagues, the Au NPs dotted thionine-functionalized CNTs and Au NPs-doped PAN-coated CNTs are used as sensing platforms and signal labels, respectively. The configuration exhibited significant sensitivity toward CEA detection due to CNTs' and Au NPs' dual-signal amplification. In another study, Wang and colleagues attempted the CEA detection using Ag NPs modified with molybdenum disulfide-coated Fe_3O_4 NPs ($Ag/MoS_2@Fe_3O_4$) as a label and similar to the ELISA method. The investigators selected Ab-conjugated $Ag/MoS_2@Fe_3O_4$ via ELISA, which resulted in the conduct of detection on magnetic GCE with the assistance of a chosen label. The configuration exhibited a 0.03 $pg \bullet ml^{-1}$ as LOD, much lower than contemporary attempts of similar times. The emergence of systematic ligand evolution through exponential enrichment paved the way for choosing aptamers to specifically recognize the high-affinity targets, leading to enhanced specificity and stability besides simpler synthesis and modification. An exclusive attempt in this reference used aptasensor based on sandwich tactics for a sensitive CEA detection. The CEA aptamer 2 (CEAapt2), dendritic Pt@Au nanowires (Pt@AuNWs), and toluidine blue together formed Pt@AuNWs-CEAapt2-Tb bioconjugate as a signal tag while Au-functionalized

GCE immobilized with CEAapt1 was used as sensing probe for capturing the CEA in a sandwich-type configuration. The configuration demonstrated a good linear response to the (0.001–80) ng•ml^{-1} CEA range with 0.31 pg•ml^{-1} LOD.

Among the notable attempts toward CEA detecting label-free electrochemical biosensors, the one by Shao and associates used a label-free immunoassay based on Prussian blue nanocubes-loaded molybdenum-disulfide nanocomposites (MoS_2-PBNCs) as a sensing platform. This platform displayed splendid electrocatalytic ability that could be applied for CEA recognition in human serum with a good resolution. The advantages offered by polymeric NMs have been incentives to improve the CEA detection resolution, wherein significant redox activity, easier modification, adequate biocompatibility, and stability are some prominent traits. Making use of these characteristics, Ji and colleagues in their 2015 work used Au NPs-doped polydopamine to modify carbon-encapsulated Fe_3O_4 NPs embedded in porous graphitic carbon NCs (Fe_3O_4@C@PGC) as CEA detection probe, with $<$0.33 pg•ml^{-1} LOD and $<$3% RSD. A detailed description could be traced to the 2017 contributions of Ji and Shao research groups. Based on relative polymers doping, Li and colleagues used Au-F127 nanospheres as an electrochemical interface for CEA detection and observed 0.5 pg•ml^{-1} LOD over a (0.001–10) ng•ml^{-1} calibration range. Owing to legal restrictions, the referred studies could be traced to the 2019 *Journal of Biosensors and Bioelectronics* contribution of Yang and associates.

6.1.8 Optical Biosensors

Among the noted attempts of optically activated biosensors for CEA detection, the 2018 study by Danesh and group used a fluorescent aptasensor based on 5,6,7-trimethyl-1,8-napthyridin-2-amine (ATMND) as a dye and a three-way junction pocket as fluorescent quenching probe. The CEA aptamer conjugate of the three-way junction pocket was stripped in CEA presence. The sensor was very effective in CEA detection with significant recovery from human serum. In another potential effort, Wang and colleagues aimed for high sensitivity and used upconversion NPs (UNPs) to fabricate a FRET-driven immunosensor using fluorescein isothiocyanate (FITC)-labeled primary Ab (Ab_1) and Au NPs-labeled secondary Ab (Ab_2) to form Au NPs-CEA-FITC-Ab complex in the presence of CEA. The sensor displayed a comparatively more universal and easy-to-use analytical view due to manifested colored and fluorescence-sensitive dual readout. More recently, black phosphorous as an emerging 2D-layered material has been studied for thickness-dependent direct band gap, high-charge carrier mobility, characteristic current on/off ratios, and, most significantly, angle-dependent transport anisotropy for an accurate CEA detection. Another worthwhile attempt by Peng and colleagues used Au NPs doped with a few layers of black phosphorous (BP-Au) as a platform for CEA detection inspired by splendid catalytic activity and low activation energy of BP-Au. On initial optimization, the SERS as a spectroscopic tool for CEA detection maximized the benefits of high sensitivity, characteristic spectroscopic fingerprint, along with nondestructive data acquisition. Using such incentives, Lin and colleagues developed a simple method for CEA detection in human serum based on Ab-adsorbed Au and

γ-Fe_2O_3@Au NPs as a probe for a (1–50) ng•ml^{-1} linear range with 0.1 ng•ml^{-1} LOD. The designed configuration exhibited efficient photoirradiation conversion to an electrical signal, high sensitivity, low cost, and simple instrumentation. In this reference, one study by Wu and colleagues used $Zn_{0.1}$ $Cd_{0.9}S$-hybridized g-C_3N_4 functionalized indium-tin-oxide slices as a photoactive matrix for CEA detection. The synergistic influence of g-C_3N_4 and NCs resulted in a high-intensity response and ultralow LOD. A 2015 attempt by Peng and colleagues reasoned the high sensitivity and wide dynamic signal response range for developing electrochemiluminescence biosensors. The developed biosensors comprised GO/carboxylated MWCNTs/Au/CeO NPs as sensing matrix for CEA detection. This platform manifested a good electron transfer, stability, and high specific surface area NCs, ensuring accurate CEA detection to ultralow LOD extent. Owing to legal restrictions, the referred studies could be traced to the 2019 *Journal of Biosensors and Bioelectronics* contribution of Yang and associates.

6.1.9 Other Biosensors

Other than electrochemical and optical mechanisms, several biosensors have been reported for CEA detection. For instance, Chu and colleagues demonstrated a FET-driven rapid and movable probe, having Ab-coated AlGaN/GaN high electron mobility transistors as detection matrix. The configuration exhibited good stability and selectivity in human serum. In another potential effort, Liu and colleagues used an ultrasensitive lateral flow immunoassay accommodating MNPs as a CEA determination probe. Yet another significant attempt toward POC CEA detection in human serum by Jiang and colleagues used glucose oxidase (GOx)-entrapped Au hollow microspheres (AuHMs) as a signal label, working through a quantitative pH determination. With the GOx-AuHM labeling, the sensor accurately tracked the CEA over a wide linear range, with 0.062 ng•ml^{-1} LOD.

4. **Squamous cell carcinoma antigen screening**: Aggravated SCCA activity remains a noted hallmark of LC, cervical squamous cell carcinoma, and hepatocellular carcinoma, making it hereby very urgent to develop sensitive and robust recognition assays. The advances in electrochemical and optical biosensors along with the emerging use of NMs are briefed next.

6.1.10 Electrochemical Biosensors

The shape and size-dependent physical and chemical properties of NMs have been the authentic assets for their enzyme equivalent functioning in the efficient design of electrochemical biosensors for SCCA detection. Making use of such attributes, Wang and colleagues prepared the composites of Au@Ag@Au NPs as enzyme mimetic tags in an attempt to attain better stability and replace the H_2O_2 catalytic response. After the immune response, the biosensor exhibited 0.18 pg•ml^{-1} LOD and a 0.5 pg•ml^{-1} to 40 ng•ml^{-1} wide detection range for SCCA. With an intent to maximize the stability and sensitivity, Liu and colleagues made a range of NCs using graphene having β-cyclodextrin-loaded graphene sheet (CD-GS) as a sensing platform owing to high supramolecular recognition of CD with GS (prevents GS

stacking besides improving its biocompatibility) and the ternary hollow Pt/Pd-Cu nanocube-fastened 3D graphene scaffold as a receptive label. Using this sensing platform and sensitive label, the investigators fabricated a controlled release system-driven labeled biosensor to detect SCCA. In furtherance, Ma and colleagues fabricated a sandwich-like biosensor having β-cyclodextrin-functionalized Au-anchored SiO_2 (CD-Au@SiO_2) as a label and primary antibody, Ab_1 restricted Au electrode as a sensing probe for an ultrasensitive SCCA screening. The investigators used 1-methyl-1H-benzimidazole-functionalized mesoporous SiO_2 to encapsulate methylene blue (MB) with CD-Au@SiO_2 as a regulator and finally entrap the arbitrarily modified secondary antibody, Ab_2. MB was released from MBI-MS at <7 pH, wherein the SCCA and functional Ab_2-participated in an immunological reaction. The detection was pursued distinctly within (0.001–20) ng•ml^{-1} with 0.25 pg•ml^{-1} LOD. Owing to legal restrictions, the referred studies could be traced to the 2019 *Journal of Biosensors and Bioelectronics* contribution of Yang and associates.

For label-free electrochemical sensing of SCCA, a 2016 study by Li and group used icosahedral Au NCs as carriers for rapid SCCA detection that were subsequently integrated to Au NPs-anchored GCE through 1,3-di-(3-mercaptopropyl)-imidazolium bromide (DMIB). The sensor exhibited a good response with 12.6 pg•ml^{-1} as LOD. A further significant attempt by Gao and associates toward fabricating a label-free electrochemical biosensor for screening SCCA used Fe_3O_4 as a nanocontainer and aminated polystyrene microspheres (APSM) as a molecular gate to achieve toluidine blue encapsulation. The optimized configuration exhibited an amplified signal as inferred through square wave voltammetry analysis for released TB, giving a correlated SCCA quantity.

6.1.11 Optical Biosensors

The recent past has witnessed significant developments in the development of photoelectric immunosensors using composite-grade NPs owing to their exceptional chemical inertness and robust action mechanism. In one such noted attempt, Wu and colleagues designed magnetic GO (Fe_3O_4@GO) as a sensing matrix and Au NPs/carbon nitride (C_3N_4) as a signal label to form an "in-electrode"-type electrochemical biosensor for SCCA detection. The Fe_3O_4@GO enabled an effective capture of the primary antibody, Ab_1, to amplify the recognition signal while Au NPs/carbon nitride (C_3N_4) enhanced the secondary antibody, Ab_2, loading capacity besides providing a high conductivity for an improved ECL intensity. The (0.001–10) ng•ml^{-1} detection range and 0.4 pg•ml^{-1} LOD argued well for efficient SCCA detection by the prepared immunosensor. Using the label-free PEC approach, Ye and colleagues used $MoSe_2$ nanosheets with photocurrent intensity and hollow Au nanospheres (HGNs) as a sensing platform for latent SCCA detection. Because of the HGNs-$MoSe_2$, NC-enhanced photocurrent intensity and improved binding site activity, the fabricated sensor exhibited a 0.21 pg•ml^{-1} LOD for SCCA. Moving on to POC devices, Lin and colleagues 2018 fabricated a naked eye colorimetric immunoassay for SCCA detection using Au nanobiopyramid-functionalized Ag nanorods (Au NBP@Ag) as carriers. The carriers were designed to modify the secondary antibody, Ab_2, and amplify the detection signal. The sensor exhibited a

good linear relationship (2.5–105) ng•ml^{-1} with 2.5 ng•ml^{-1} LOD (for naked eye) and 0.85 ng•ml^{-1} (for spectrometer). Owing to legal restrictions, the referred studies could be traced to the 2019 *Journal of Biosensors and Bioelectronics* contribution of Yang and associates.

6.1.12 Carcinoma Antigen 125 Detection

A shift or change in carcinoma antigen 125 (CA 125) concentration reveals the stage of LC, the reason for which investigators are interested to detect <35 U•ml^{-1} extent of this biomarker. The progress regarding sensing attempts made for CA 125 detection is described next.

6.1.13 Electrochemical Biosensors

The simple, easy-to-operate, highly sensitive, and rapid detection procedures of electrochemical immunoassays have been the basis for manifold electrochemical biosensors being reported for CA125 detection. In one of these past decade attempts, Torati and colleagues reported one-step electrochemical CA125 detection using layered Au nanostructures as a platform that on being coupled to CA125 binding sites generated a significant electron transfer to amplify the detection signal. The analysis of this sensor using differential pulse voltammetry revealed a splendid linear response with 5.5 U•ml^{-1} LOD. A modification in the sensing platform via incorporating polymer–metal complex, PANI in conjugation with Au NPs and catalytic NMs was attempted by Zheng and colleagues, who developed an amperometric immunosensor for CA125 detection. The analytical response was obtained in the (0.01–5000) U•ml^{-1} range with 4.4 mU•ml^{-1} LOD. Still another study developed a microflow biosensor using thin film-modified Au array microelectrodes prepared by electrocatalyzed polymerization of anthranilic acid to IDAs as a sensing platform. The platform was used to ascertain the progress of immunoreactions through electrochemical impedance spectroscopy (EIS), whereby the complex detection transformation was simplified.

6.1.14 Optical Biosensors

The first major last decade attempt using optical biosensors-mediated CA125 detection was the investigation of Al-ogaidi and colleagues, who designed a CL resonance energy transfer biosensor using graphene quantum dots (GQDs) as a detection platform and HRP-labeled secondary Ab as the probe. The immunosensor retrieved an energy transfer between reactive oxygen species (generation of HRP-Ab_2 catalyzed H_2O_2) and luminal on the formation of CA125-immobilized Ab_1 and HRP-Ab_2 complex. The designed configuration exhibited a (0.1–600) U•ml^{-1} detection range and a 0.05 U•ml^{-1} LOD. In fluorescence immunoassays, GQDs and carbon dots are preferred as immune tags for enhanced DS, resulting from the adequate and uniform surface modification, biocompatibility, and photobleaching stability. A potential attempt by Tsai and colleagues focused on the fabrication of GQDs labeled with Ab_1 as a sensing platform and fluorescent NPs conjugated with Ab_2 as a detection probe. Another significant attempt by Hosu and accomplices proposed a colorimetric smartphone-driven immunoassay working through

detection of the grayscale index of (Ab_1-Ca125-Ab_2@Au)-sandwiched immunocomplex, stained by Ag, for robust CA125 detection. Owing to legal restrictions, the referred studies could be traced to the 2019 *Journal of Biosensors and Bioelectronics* contribution of Yang and associates.

6.1.15 Other Biosensors

Among the noted attempts based on lab-on-a-chip and POC, CA125 detection is the study by Nunna and colleagues, who fabricated a biosensor working via microfluidic flow conditions for monitoring the capacitive sensor. Another effort in this direction was made by Mansouri and associates, who introduced a flexible FET-type aptasensor using MWCNTs/rGO as a CA125 sensing platform. Another significant contribution was the study of Ju and colleagues, who reported an EDFM-DM sensor using Ag NPs as a fluorescent-devoid CA125 detection probe. Both (this and previous) biosensors exhibited a good linear range of response with ultralow LOD toward CA125 detection in clinical samples and biological fluids. Referred studies could be traced to the 2019 review article by Yang and associates featured in the *Journal of Biosensors and Bioelectronics* since the references as per legal bindings for the present compilation cannot exceed 50.

5. **Tissue polypeptide antigen**: TPA shows abnormal activity in rapid increments in malignant tumors as there is a rise in TPA expression amidst mitosis (cell division of increasing cell populations). This is the reason for considering TPA as an important factor in the auxiliary diagnosis, the basis on which TPA sensing is done to monitor LC prevalence. The first notable effort in this regard is the study of Wang and colleagues, who reported a sandwich configuration of the biosensor using graphene sheets as a signal transfer platform and Pd–Pt bimetallic NCs-labeled Ab as a probe for H_2O_2 catalysis. The developed sensor showed a DS in the range of pg•ml^{-1}. Interestingly, Pd–Pt combines exhibited superior catalytic efficacy compared with any single NC besides exhibiting higher stability than enzyme-tagged Ab_2. Another study by Wang and colleagues used multifunctional graphene NCs (Au@MGN) through Au NPs absorption as nano-Fe_3O_4@GO with significant electrochemical property and biocompatibility. Subsequently, the investigators fabricated an electrochemical immunosensor using Au NPs-modified GCE as a platform and Au@MGN as a TPA detection probe. The sensor exhibited a wide calibration range (from 10 fg•ml^{-1} to 100 ng•ml^{-1}) and 7.5 fg•ml^{-1} as LOD. The same research group of 2016 fabricated an immunoassay using 3D-ordered macroporous Au films as matrix and bifunctional, nano-raspberries as labels for TPA detection. It was ascertained that these bifunctional, nano-raspberries exhibited a higher peak current reduction (compared with Au and Pt NPs) due to remarkable electron transfer ability and intense catalytic activity toward H_2O_2. Referred studies could be traced to the 2019 review article by Yang and associates featured in the *Journal of Biosensors and Bioelectronics*.

Table 13 Conventional techniques for breast cancer diagnosis with concurrent concerns

Technique	Limitations
Mammography	Low sensitivity and specificity Sensitivity falls on rising tissue density Unable to ascertain tumor in dense and rigid tissue at the initial stage Frequent false-positive outcomes Low-energy X-rays could cause mutations Disintegration of tumor tissue amidst analysis may result in metastasis
Biopsy	May untrace the tumor cells Needless and elaborate surgery May result in metastasis Needs talented expertise Suited as a later-stage confirmation Expensive
MRI	Unable to detect ductal and lobular carcinoma Costly
Sonography	Requires expertise for real-time monitoring Less sensitive and relatively expensive
Fluorescence in situ hybridization (FISH)	Provides semiquantitative results Separates the sufferers as biomarker positive or negative groups
ELISA	Elaborate Costly Unresponsive to low-level expression markers Intrinsic analyte color may give false results
Radioimmunoassay (RIA)	Radioactivity risk Complex procedure Elaborate Needs skilled manpower
Immunohistochemistry (IHC)	Complicated technique Time-consuming Requires skilled manpower

6.1.16 Breast Cancer

Recognized as the most fatal disorder among females, BC contributes to ~23% of global cancer cases globally, typically accounting for the second largest extent deaths among all cancer-related deaths (DeSantis et al. 2014). The scenario is more precarious in developing and underdeveloping economies, where lacking clinical facilities and missing awareness aggravate the treatment recourses. The involvement of various factors in BC onset is the reason for diversified symptoms depending on the specific size and type of tumor. Accordingly, the treatment varies and requires timid diagnosis, efficient remedial procedures, and post-medication cautions to nullify a recurrence. Predicted as being carried by every one in eight US women with staggering 39,620 deaths reported in 2013 itself, the BC menace has been dreadful (DeSantis et al. 2014). Over the last few decades, BC treatments have seen consistent progress, facilitating prompt recognition with reduced mortality. It is

pertinent to mention here that BC classification can be made using morphological or immunohistochemical markers.

Conventional procedures for BC diagnosis include mammography, biopsy, MRI, sonography, molecular breast imaging, thermography, etc. (Table 13). These methods are steadfast and can screen (80–90)% tumors. Besides these methods, biomarker expressions are also screened such as ELISA, radioimmunoassay, and immunohistochemistry. Despite being efficient and enjoying a majority level of patient confidence, all these methods are associated with certain limitations, presumably due to unpredictable mutational reasons that remain nonprogrammed in the diagnostic probes. False-positive (negative) results obscure the situation through error-prone analysis and needless biopsies. So, present research attempts to counter BC mortality are dedicated to the expansion of detection methods that are highly sensitive, noninvasive, and amenable for POC diagnosis. The salient attributes of biosensors include their sensitivity, specificity, and cost-effectiveness besides a quick response for the shortest appraisal of physiological fluids (including blood, serum, urine, saliva, milk, etc.) in a non-patient-sensitizing manner. For the subsequent sections about BC, readers are suggested to refer the 2017 *Biosensors and Bioelectronics* contribution of Mittal and colleagues and the 2019 Elsevier book Chapter 3 by Pereira and accomplices.

6.1.17 Need for Biosensor-Facilitated Breast Cancer Diagnosis

The biosensor for BC diagnosis works through a distinctive screening of biomarkers (to be screened target molecule) using the coordinative functioning of bioreceptors (recognizing element) and a compatible biotransducer. The biomarkers corresponding to BC are depicted in Fig. 21, distinguished into stage and

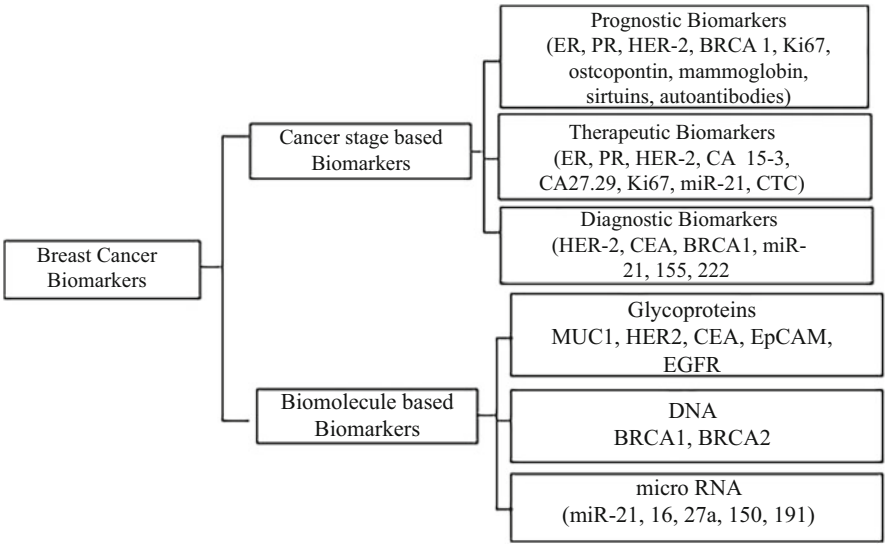


Fig. 21 Classification of breast cancer biomarkers

biomolecules based. Typical biomarkers prevailing on cell surface gradually shed off extracellular domains (ECDs) in the serum are usual analytes. It is pertinent to mention here that the biomarkers vary in their specificity concerning the tumor concerned and may exhibit varied expression levels corresponding to different tumor stages.

So, multiplexing the biomarkers at a uniform screening platform is likely to provide better inference. Studies identifying these indications (individual or in combinative mode) illustrate a simplification via classifying the tumor cells that ultimately provides the prognosis information. For distinct recognition of a biomarker, selecting a specific biotransducer regime is a prerequisite, which in turn depends on the biochemical signal generated by implicit biomarker–bioreceptor interaction. For instance, change in H^+ concentration is ascertained through potentiometric biotransducer, gain or loss of electrons is made through the amperometric transducer, light emission/absorption/fluorescence/reflectance is made via optical screening, and a mass variation is perceived by the piezoelectric biosensor. Finally, some enhancers (usually NMs or high adsorptive properties harboring reagents) are needed to intercept a lower extent of bioreceptor–biomarker interaction.

There are several biomolecules such as cell surface proteins, mutated genes, and microRNAs that vary in the expressed in/on tumor cells and are implicit indicators of tumor progression. Such biologically pertinent entities are known as biomarkers, which are typically required to be easily extractable from the physiological fluids of the sufferers in a nonsensitizing procedure. Like all other cancer biomarkers, those for BC also exhibit dual-classification modes as stage-dependent and overexpressed biomolecules (Fig. 21). From a diagnostic viewpoint, biomolecules-based biomarkers have a greater significance, although there always remains a definitive correlation between diagnostic and prognostic significance of a biomarker. For instance, human epidermal growth factor receptor 2 (HER-2), and estrogen and progesterone receptors are recognized as diagnostic as well as prognostic biomarkers. The following sections discuss the biomolecules-based biomarkers that are recognized as target molecules in biosensing.

1. **Glycoproteins**: These biomarkers mainly comprise the surface-bound carbohydrate–protein combinative molecules, such as HER-2, Mucin1 (MUC1), and CEA, epithelial cell adhesion molecule (EpCAM), and EGFR. In general, these are unregulated and result in cancer cell proliferation via growth factor generation, which alters the MAPK, PI3K/Akt pathways besides inducing metastasis. Among the several BC-sensitive glycoproteins, HER-2 and MUC1 are the most standardized diagnostic and prognostic biomarkers. HER-2 is a proto-oncogene encoding trans-membrane glycoprotein, the overexpression of which is detected in (20–30)% of BC cases. HER2-positive BC is the most aggressive of all known BC forms. The HER-2 receptors comprise extracellular ligand-binding domain that undergoes dimerization on ligand binding and an intracellular tyrosine kinase domain that undergoes phosphorylation in response to dimerization. This phosphorylation henceforth activates the manifold receptors to pave the way for downstream signaling cascade pathways, viz., APK, PI3/Akt, etc.

MUC1 is a glycoprotein that guards against pathogenic infections and is encoded by the MUC-1 gene. It is generally expressed at the apical surface of epithelial cells in several organs including the breast. However, in BC cells, MUC1 is overexpressed over the whole cell surface.

2. **DNA biomarkers**: Mutations in oncogene result in tumor aggravation contrary to those of tumor suppressor genes that fail in their functions and are usually associated with tumor incidence and are widely used as recognition biomarkers. Epigenetic alterations in nuclear and mitochondrial DNA are also recognized as indicators of BC progression. For BC diagnosis, BRCA1 and BRCA2 genes are the typical and largest probability exhibiting DNA biomarkers, which are the tumor suppressor genes articulated in normal cells and help in repairing the double-stranded DNA breaks or induce death, if these are beyond repairable extent. They also regulate the cell cycle checkpoints and cell division. Thus, these collectively play a decisive role in maintaining genome integrity. Mutations in BRCA1 and BRCA2 genes are usually associated with an aggravated BC threat that is noticed in nearly (21–40)% of inherited BC cases. Expression of BRCA1 protein is reported as reduced in 30% of sporadic BC cases. The relative extent of BRCA1 protein downregulation depends on the BC severity and is inversely related to BRCA2 protein expression, a characteristic that is used for the diagnosis of sporadic BC. Thus, BRCA2 protein can be screened as a prognostic and diagnostic BC biomarker.
3. **MicroRNAs**: MicroRNAs (miRNAs or miRs) are small noncoding RNA fragments regulating a characteristic gene expression. These fragments act by masking the actual function of the underlying genome region, whereby their upregulation and downregulation both are considered pertinent diagnostic markers. Several miRs are up- and downregulated in cancers, but miR-21 is exclusively screened for in BC diagnosis owing to its higher (87.6%) sensitivity and specificity (87.3%) in the initial stage compared to other biomarkers (CEA and CA15-3). The miR-21 is overexpressed in blood plasma and cancerous tissue compared to normal breast tissue due to which it is significant as a diagnostic and therapeutic biomarker. The aggravated expression of miR-21 is associated with increased metastasis of BC via MAPK pathway stimulated HER-2 activity. Despite being established as a stable and noninvasive biomarker in BC diagnosis, it exhibits certain limitations, including sequence homology with related RNAs, the prevalence in other (except BC) cancers, and low serum occurrence. Such issues, therefore, limit the miR use as biomarkers. To overcome these arbitrary hurdles, usually northern blotting and in situ hybridization are practiced for miR screening, but here also, a low sensitivity along with more time and sophisticated conduct setup curtail the biomarker suitability of miR.

   Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a highly responsive and broad-spectrum procedure for miR analysis, but it also exhibits certain limitations concerning erstwhile laboratory-bound assays. In this reference, electrochemical biosensors have emerged as breakthrough POC devices. Initiating attempts dedicated to miR detection exclusively relied on hybridization and guanine oxidation. Thereby, miR-21 is detected through its

hybridization by monitoring its hybridization with a complementary probe and has been investigated. Of late, a consensus about miR expression levels revealed decreased miR-21 expression (to femtomolar extents) using iridium (III) complex with miR-21 recognizing G-quadruplex.

4. **Circulatory tumor cells (CTCs)**: The characteristic morphological traits of cancer cells are highly different from their normal counterparts, which also augment their screening. CTC analysis is an inference of tumor metastatic activity, the separation, and quantification of which could assist the cancer screening. Instinctive information conveyed by CTC includes the invasive extent of a particular tumor, through antitumor drug response along with the mechanism of personalized anticancer therapy. The prominent distinguished phenotypic features of cancer cells aiding in their isolation from normal cells include size, morphological prospects, and magnetic sensitivity. The quantification is rendered difficult due to their low bloodstream (0–10 per ml of whole blood) count compared to healthier and physiological constituents (e.g., 10^9 erythrocytes and 10^6 leucocytes per ml blood). Studies predict with certainty that a high tumor population could be a decisive factor as a clinically imperative phase in BC.
5. **Other biomarkers**: Certain biomarkers are specifically targeted for BC diagnoses, such as cell-free DNA, autoantibodies, and antigens such as urokinase-dependent plasminogen activator system (UPA), the plasminogen activator inhibitor (PAI), and the Thomsen–Friedenreich (TF). A brief description of such biomarkers is appended later.

6.1.18 Cell-Free DNA

Circulating cell-free tumor DNA (cfDNA) is a significant cancer biomarker, with the BC having an implicit association with excessive DNA damage caused by the apoptotic and necrotic cells. These cells prevail within (0–2000) $ng \bullet ml^{-1}$ (as DNA content) in plasma, serum, and urine. Quantitative profiling of cfDNA provides ample scope to develop noninvasive methods of BC diagnosis besides therapeutic information. Studies focused on exploring cfDNA as a BC biomarker infer a direct correlation between BC progression and cfDNA concentration. However, their use in biosensor-mediated BC detection is still in a progressive state and requires an authentic analysis of underlying mechanisms.

6.1.19 Autoantibodies

A potential role of autoantibodies in early-stage BC diagnosis has been made much later than other biomarkers. It was noticed that the malignant potency of a cancer results in Abs circulation against the cancer antigens in the serum, which can be easily used as a biomarker to ascertain the instantaneous tumor status. Autoantibodies against tumor-associated antigens (TAA) can be ascertained in the serum and saliva of the sufferers, listed as effective biomarkers for an early diagnosis. The autoantibodies reported in the BC patients against the corresponding antigens include p53, heat shock proteins (HSP- 27, 60, and 90), GIPC-1, c-myc, c-myb, cyclin D1, cyclin B1, RS/DJ-1, etc. The expressions of these have been reported as a positive correlation between the extent and cancer progression. For instance, an

Table 14 Summary of biosensors aimed at BRCA1 detection in the human serum

Transducer	Principle	LOD and linear range	Response time and shelf life
Electrochemical	Restriction of complementary DNA probe	2.104 μA•fmol^{-1}; (0.05 to 25) fmol	16 s and 6 months
Electrochemical	Complementary sequence immobilized on Au NPs inside a highly cross-linked amine-modified PEG film	1.72 fM; 50.0 fM to 1 nM	Undisclosed
Electrochemical	DNA probes conjugated to PANI/PEG nanofibers	0.0038 pM; 0.01pM to 1 nM	30 min and >10 days
Electrochemical	Zwitterionic peptides anchored to citrate doped PEDOT conducting polymer	0.03 fM	Undisclosed
Fluorescence	Carbon dots-based fluorescent passage and Au NPs for recognizing nucleotide BRCA1 sequences	(4–120) nM	Undisclosed
Colorimetric	Three spots labeled with digoxin for each detected target to amplify the typical enzymatic reading	10 fM and 10 fM to 10 nM	Undisclosed

autoantibody-based microarray has been reported for 10 respective BC tumor antigens and successfully applied to investigate the BC samples. Although detection of autoantibodies in serum is a noninvasive approach, the heterogeneous nature of BC, along with poorly understood humoral immune response, limits their frequent clinical application.

Urokinase-Dependent Plasminogen Activator, Plasminogen Activator Inhibitor (PAI), and Thomsen–Friedenreich antigens

Besides the above biomarkers, a combinatory prevalence of independent molecules is also proposed to augment the diagnostic efficacy. Urokinase-dependent plasminogen activator, PAI, and TF antigens are also reported as disease signals in pre- and postmenopausal women. However, the detection of these markers in real samples through biosensors is awaited based on which multiple novel avenues against BC diagnosis could be opened.

6.1.20 Screened Breast Cancer Biomarkers in Biosensors Accomplished Diagnosis

Tables 14, 15, 16, 17, and 18 comprise the different biosensor configurations for prompt detection of BC biomarkers. With progress in electron microscopy and sharper precision tools, robust and faster principles are being swiftly undertaken to develop devices for affordable, portable, and time-scale robust analysis without any compromise in sensitivity and specificity. Although most of the developed systems are not yet feasible for clinical or market applications, the explored analyte–probe

Table 15 ERα, PR, and CEA bioreceptor targeting biosensors for BC diagnosis

Biomarker	Transducer	Principle	LOD and linear range	Response time and shelf life
Erα	Optical	The hollow core of photonic crystal fiber with anti-ER-labeled primary and secondary Abs	0.4 μg•ml^{-1}	Undisclosed
PR	Electrochemical	Aptamer on Au electrode and iron redox probe settings	0.90 μg•ml^{-1}, 10–60 ng•ml^{-1}	40 min and undisclosed
CEA	Colorimetric	Au NPs and a few layers of black phosphorous hybrid	0.20 pg•ml^{-1}, 1–10^{4} pg•ml^{-1}	Undisclosed
CEA	Chemiluminescence	CEA aptamer conjugates with hemin aptamer through 1,1′-oxalyldiimidazole	0.58 ng•ml^{-1} 0–200 ng•ml^{-1}	30 min and undisclosed
CEA	Colorimetric	Au NPs as anti-CEA Ab carriers labeled with biotin	48 pg•ml^{-1}; 0.05–50 ng•ml^{-1}	15 min and undisclosed
CEA	Electrochemical	Paper-based microfluidics immunodevice	0.01 ng•ml^{-1}	Undisclosed
CEA	Fluorescence	FRET between upconverting NPs and Pd NPs	1.7 pg•ml^{-1}/(4–100) pg•ml^{-1}	Undisclosed

Note: Attainment of nano- and microscale detection range through different probe configurations define the diversity of biosensor functioning

Table 16 HER2 bioreceptor targeting biosensors for breast cancer detection

Transducer	Principle	LOD and linear range	Response time and shelf life
Electrochemical (amperometric)	Sandwich immunoassay working through nanobodies	Undisclosed and (1–200) $\mu g \cdot ml^{-1}$	(2–20) min and >3 weeks
Electrochemical	DNA-derived electric current with DNA self-assembly for signal intensification	0.047 $pg \cdot ml^{-1}$ and (1–100) $pg \cdot ml^{-1}$	Undisclosed
Colorimetric	HER2 Abs anchored Au NPs-loaded liposomes	5 Sk-Br-3 cells and undisclosed	2 h and undisclosed
Electrochemical (amperometric)	Modified Au NPs and graphene oxide loaded on GCEs	0.16 nM, (0.37–10) nM	Undisclosed
Electrochemical	Sandwich aptasensor using molybdate to generate an electrochemical current	Undisclosed and (0.01–5) $ng \cdot ml^{-1}$	Undisclosed
Electrochemical	Organic-electrochemical transistor	Undisclosed and (10^{-14} to 10^{-7}) $g \cdot ml^{-1}$	Undisclosed
Electrochemical (voltammetry)	Reduced graphene oxide-chitosan film as an electrode with MB as a redox probe	0.21 $ng \cdot ml^{-1}$, (0.5–2) $ng \cdot ml^{-1}$	Undisclosed
Electrochemical	Immunosensor with hydrazine and aptamer-conjugated Au NPs	37 $pg \cdot ml^{-1}$, 1 $ng \cdot ml^{-1}$ to 10 $\mu g \cdot L^{-1}$	Undisclosed
Electrochemical (voltammetry)	Anti-HER2 Abs conjugated with FeO NPs on Au electrode	0.995 $pg \cdot ml^{-1}$, 10 $ng \cdot L^{-1}$ to 10 $\mu g \cdot ml^{-1}$	Undisclosed
Photoelectrochemical	ZnO/graphene composite and S6 aptamer on a portable indium-tin-oxide microdevice	58 $cells \cdot ml^{-1}$, (10^2 to 10^6) $cells \cdot ml^{-1}$	20 min and >2 weeks
Electrochemical (EIS)	Charge transfer resistance of an Fe redox probe changes with Ab-bound protein extent	7.4 $ng \cdot ml^{-1}$, (10–110) $ng \cdot ml^{-1}$	35 min and undisclosed
Electrochemical	The immobilized polycytosine DNA sequence in an Au NPs matrix	0.5 $pg \cdot ml^{-1}$, (1–1000) $pg \cdot ml^{-1}$	Undisclosed
Electrochemical	Aptamer-modified interdigitated Au electrodes	1 pM; 1 pM to 100 nM	Undisclosed
Electrochemical	Inkjet-printed 8-electrode array, needing biotinylated Ab and polymerized HRP labels	12 $pg \cdot ml^{-1}$ and undisclosed	15 min and undisclosed

configuration could steadfast the BC diagnosis in near future. The following sections discuss the marker-specific studies and advances chronologically.

BRCA1: Manifold configurations of biosensors have been proposed and reported for early BRCA1 detection (Table 14). To begin with, Tiwari and colleagues demonstrated an electrochemical biosensor using chitosan-co-polyaniline as a

Table 17 The different biosensor configurations used for screening the characteristic BC biomarkers

Biomarker	Transducer	Principle	LOD and linear range	Response time and shelf life
Mucin 1	Electrochemical	Aptamer//cell/aptamer sandwich array on an electrode surface	100 cells•ml^{-1} (10^2 to 10^7) cells•ml^{-1}	Undisclosed
Mucin 1	Electromagnetic	Aptamer-functionalized Au nanorods	100 cells•ml^{-1} (10^2 to 10^5) cells•ml^{-1}	30 min and undisclosed
Mucin 1	Electrochemical (voltammetry)	Polyadenine-aptamer-functionalized Au NPs/graphene oxide hybrid	8 cells•ml^{-1} and (10–10^5) cells•ml^{-1}	40 min and undisclosed
Mucin 1	Electrochemical	Biotinylated aptamer immobilized on Au NPs-graphene oxide-PEDOT composite	0.031 fM, 3.13–31.25 nM	15 min and 14 days
CA 15-3	Optical	Abs immobilized via surface standard amine coupling on an optofluidic ring resonator	1 unit•ml^{-1} and undisclosed	20 min and undisclosed
CA 15-3	Optical	Cysteamine-capped cadmium sulfide QDs	0.002 kU•L^{-1} and undisclosed	15 min and undisclosed
CA 15-3	Electrochemical (voltammetry)	Detection of seven tumor markers using alkaline phosphatase-based competitive immunoassay for hydroquinone screening	0.7 U•ml^{-1} and (1.2–3.7) U•ml^{-1}	Undisclosed
CA 15-3	Electrochemical	Nanoporous Au/graphene hybrid platform combined with HRP	5•10^{-6} U•ml^{-1}, (2•10^{-5}-40) U•ml^{-1}	Undisclosed
CA 15-3	Electrochemical	Label-free highly conductive N-doped graphene sheets modified electrode	0.012 U•ml^{-1} and (0.1–20) U•ml^{-1}	Undisclosed
CA 15-3	Electrochemical	Functionalized graphene with 1-pyrenecarboxylic acid sensing probe and MWCNTs with ferritin labels	0.009 U•ml^{-1} and (0.05–100) U•ml^{-1}	Undisclosed
CA 15-3	Electrochemical	Electrically conducting poly (toluidine blue) as synthetic receptor film	0.10 U•ml^{-1} and (0.10–100) U•ml^{-1}	Undisclosed

Note: LOD, working range, response time, and probe shelf life are the decisive performance defining criteria

Table 18 miR-21 and miR-155 target attempts toward a prompt breast cancer diagnosis

Biomarker	Transducer	Principle	LOD and linear range	Response time and shelf life
miR-21	Electrochemical	Probes conjugated to a pencil graphite electrode	1.0 mg•ml^{-1}	Undisclosed
miR-21	Electrochemical	Two supplementary probes that self-assemble to form 1D DNA concatemers	100 aM; 100 to 10^5aM	Undisclosed
miR-21	Electrochemical	MB as a redox indicator	84.3 fM; 0.1–500 pM	60 min and undisclosed
miR-21	Electrochemical	Hybridization to a specific biotinylated DNA probe restricted on magnetic beads	0.04 pM; 0.1–500 pM	30 min and undisclosed
miR-21	Fluorescence	2-Aminopurine probe in conjunction with a G-quadruplex structure	1.48 pM; undisclosed	Undisclosed
miR-21	Electrochemical	Probe engineered with a pyrrolidinyl peptide nucleic acid/PPy/Ag nanofoam	0.20 fM and (0.20–10^6) fM	Undisclosed
miR-21	Electrochemical	Target stimulated glucose release from propylamine-functionalized mesoporous silica NPs	19 pM; (50–5000) pM	Undisclosed
miR-155	Electrochemical	Graphene oxide sheet on GCE surface with thiolated probe-functionalized Au nanorods	0.60 fM; (20–8000) fM	Undisclosed
miR-155	Electrochemical	Immobilization of anti-mi-R-155 on Au SPE	5.7 aM; (10–10^9) aM	Undisclosed
miR-155	Colorimetric	Covalent conjugation of a DNA probe to negatively charged Au NPs	100 aM; (100 to 10^5) aM	Undisclosed

sustainable support platform while being applied on an indium-tin-oxide support. The study involved designing a probe with BRCA1-associated cDNA sequences that were immobilized over a surface and produced an electrochemical response in the presence of a single-stranded DNA. The configuration revealed 0.05 fmol as LOD with splendid sensitivity and reproducibility to facilitate early-stage BC detection. In another attempt, Wang and colleagues also demonstrated an electrochemical biosensor aimed to screen the BRCA1 in the serum to an extent as low as 1.72 FM. The configuration comprised a label-free DNA sensor made via GCE modification carrying highly cross-linked PEG film containing $–NH_2$ groups. This sensor was further tailored using Au NPs, conferring an outstanding sensitivity.

Another significant attempt was made by Hui and associates, who used PAN/PEG nanofibers as probe materials to detect BRCA1. The fabricated configuration facilitated BRCA1 detection in the human serum without getting affected by nonspecific

adsorption in the complex media with nanofibers and conferred antifouling abilities for a significant immobilization-driven probe capture.

Through another attempt, Wang and associates used zwitterionic peptides modified with a citrate polymer-doped poly(3,4-ethylenedioxythiophene (PEDOT), which imparted an antifouling ability along with enhanced electrical conductivity to make way for the binding of a suitable DNA probe. This configuration exhibited a much lower LOD (0.03 fM) compared to the earlier one (1.72 M) demonstrated by the same research group. Henceforth, another notable attempt by Zhong and colleagues comprised a fluorescent, dual-channel biosensor configuration based on carbon dots and Au NPs, working through a hairpin structure. The working involved a specific binding of BRCA1 RNA/DNA targets to its complementary sequence on Au NPs followed by a subsequent release from carbon dots, resulting in a positive fluorescent signal with a linear response, ranging within (4–120) nm. Another attempt by Yang and associates demonstrated a sandwich-like biosensor that worked through a magnetic bead platform. The configuration involved a tetrahedral probe, having its three vertices labeled with digoxin while the fourth was labeled with a detection probe. The enzyme-labeled antidigoxin Ab thereby possessed three distinct regions for binding each detection probe, which together facilitated signal amplification. The configuration distinguished the DNA sequences with only one base mismatch and provided PCR-grade products.

ERα: The sole attempt toward ERα detection-mediated BC screening was made by Padmanabhan and colleagues, who used an optical transducer for a volumetric detection of proteins, to the extent as low as 50 nL (Table 15). The configuration used a hollow core-photonic crystal fiber working via TIR mode, allowing a fluorescence-driven green and red response on being recognized by secondary Ab.

Progesterone (PR): The summary of studies dedicated to using PR detection-enabled BC screening is described in Table 15. The first notable effort was made by Jimenez and colleagues, who selected a PR aptamer through systematic ligand evolution via significant enrichment (SELE) to develop a label-free aptasensor having an enhanced signal gain, monitored using EIS. The conformational change of aptamer immobilized on Au electrode on PR binding revealed an enhanced resistance to electron transfer on an iron standard redox probe with (10–60) ng•ml^{-1} linear PR detection and 0.90 ng•ml^{-1} LOD.

Developments toward CEA detection-enabled BC diagnosis are summarized in Table 15, wherein the first notable attempt by Peng and colleagues used the catalytic attributes of a few layers of black phosphorous modified through a concurrent generation of Au NPs toward 4-nitrophenol that was screened using colorimetric assays. This catalytic activity was reversibly suppressed in an Ab's presence but was reactivated on CEA inclusion. The 0.20 pg•ml^{-1} as LOD and 1 pg•ml^{-1} to 10 μg•ml^{-1} as linear detection range argued well for distinguished sample analysis. In another significant attempt, Khang and associates fabricated a chemiluminescence aptasensor having dual DNA aptamer for competitive CEA and hemin binding within 30 min at RT. Subsequently, Amplex Red and H_2O_2 were added to generate resorufin, which depended on the HRP concentration equivalent for mimicking G-quadruplex DNAzyme formed on hemin and dual DNA-binding interaction.

The detection was made through the development of red light on 1,-1′-oxalyldiimidazole addition to the analysis cell, decreasing the intensity with enhanced CEA concentrations.

Yet another significant attempt by Liu and colleagues demonstrated a colorimetric enzyme immunoassay having Au NPs as carriers for HRP-labeled anti-CEA detection Ab and magnetic microparticles as sustaining substrates. The generated complex resulted in an optical signal for an improved sensitivity compared to ELISA-detected CEA. A subsequent attempt by Wu and associates demonstrated a sandwich immunoassay having a secondary Ab facilitated the growth of the long-chain polymeric material to provide adequate HRP-binding sites. The growth of polymeric material played a key role in signal amplification, with a direct positive correlation of secondary Abs bound to the support and the generated electrochemical signal by the HRP-O-phenylenediamine-H_2O_2 system. The support was formed by a carbon electrode printed on a paper-based microfluidics electrochemical immune device. Another attempt by Li and associates demonstrated a FRET-based biosensor using UNPs and Pd NPs. With aptamer bound to UNPs, the proximity of Pd NPs to the aptamer quenched the UNPs fluorescence. In the presence of CEA, the aptamer preferentially combined with CEA and yielded conformational changes to weaken its interaction with Pd NPs, facilitating recovery of fluorescence signals. The configuration enabled an ultrasensitive CEA detection from the diluted human serum within (4–100) pg•ml^{-1} linear range and 1.7 pg•ml^{-1} LOD.

HER2: Among the most targeted BC biomarker, HER2 has been extensively screened as a BC progression indicator, and these attempts have most actively used the electrochemical configurations (Table 16). In one of the earliest attempts of the last decade, Patris and colleagues designed a sandwich immunoassay based on nanobodies aimed to recognize another HER2 epitope on SPEs. The capture nanobody was immobilized on the carbon-working electrode while the detection of Ab was HRP-conjugated. Upon detection, the signal corresponded to p-quinone electroreduction, generated by the HRP at SPE in the presence of hydroquinone and H_2O_2. In another experimental setting, Shen and associates developed a self-assembly-driven DNA intensification biosensor capable of generating electric current. The HER2 aptamer served the purpose of both ligand (for recognition) and signal-generating reporter on a sandwich format. The sensor LOD was 0.047 pg•ml^{-1} with a (1–100) pg•ml^{-1} detection range. In the next major attempt, Tao and associates designed a colorimetric biosensor having a probe with HER2 Abs anchored on liposomes loaded with Au nanoclusters. The specific intent of using Au nanoclusters was their intrinsic peroxidase ability that resulted in the color change of solution on reacting with 3,3′,5,5′-tetramethylbenzimide in the presence of H_2O_2. The configuration enabled the screening of HER2-positive BC cells in human serum and BC tissue with an LOD of even five cells.

- Yet another significant attempt by Saeed and colleagues utilized Au NPs with a short harmonizing HER2 sequence, being covalently bonded to graphene oxide-modified GCE. Binding of HER2 to Au NPs with the involvement of an additional short stretch of DNA modified via HRP-hybridized-free HER2 sequence generated the corresponding electrochemical signal (binding of TMb with H_2O_2).

Another elegant attempt by Hu and accomplices used a HER2-specific aptamer as a ligand to capture HER2 by generating a redox-modulated current signal. The intensity of this current varied with the progress of the aptamer–molybdate phosphate moieties reaction, producing a current that varied directly with HER2 concentration in the (0.01–5) ng•ml^{-1} range. A separate study by Fu and colleagues reported an organic electrochemical transistor-based biosensor for intercepting the electrochemical actions on gate electrodes to an extent of 10^{-14} g•ml^{-1}. The Au GATE electrode was engineered with a capture-specific polyclonal anti-HER2 Ab, enabling the detection via HRP-bound secondary Ab. The current was generated as a response to the H_2O_2 and HER2 interactions.

- A subsequent attempt line is the effort of Tabasi and colleagues, who fabricated an ultrasensitive electrochemical aptasensor using graphene and chitosan film as a compatible electrode material for aptamer binding. Readers must note here that graphene and chitosan combination has been investigated significantly for a thin film formation, along with negligible nonspecific reaction and accompanied biocompatibility of chitosan. The HER2 interaction with the aptamer resulted in conformational variations that were attributed to a concentration-dependent higher signal being generated by the electrochemical probe MB. Henceforth, Zhu and colleagues fabricated a sandwiched configuration of biosensor, wherein a probe comprised of an immobilized Ab on a nanocomposite having Au NPs capped with 2,5-bis(2-thienyl)-1H-pyrrole-1-(p-benzoic acid) unswervingly on a bare electrode surface. The detection was accomplished using a hydrazine-Au NP–aptamer bioconjugate, having hydrazine as reactant bound to the Au NPs that contained Ag for the moderation of signal intensification. A turning point aspect of this study was the silver staining of target cells that exhibited a black appearance that could be easily viewed through a microscope, thereby making the clinical analysis of tumor cells a robust task.
- In furtherance, Emami and accomplices demonstrated a label-free immunosensor, wherein anti-HER2 Abs are conjugated with FeO NPs, which were further spread over an Au electrode surface. The sensor exhibited a significant response to screen as low as 0.995 ng•ml^{-1} HER-2 with 5.921 μA ml•ng^{-1} sensitivity. A succeeding attempt is the effort of Liu and associates, who demonstrated a PEC biosensor for screening SK-Br-3, an HER2-positive cell line. The investigators used a high photoelectric signal of ZnO along with graphene's splendid charge transportation and separation and S6 aptamer's specificity to target SK-Br-3 cells. Next in line is the study of Akran and coworkers, who developed an electrochemical immunosensor for HER2 screening via the preparation of carbon paste electrodes using graphite powder, multiwalled CNTs, an ionic liquid, paraffin, and the entire assembly functionalization with Au NPs (through electrodeposition). The optimized configuration exhibited a charge transfer resistance that increased linearly with increasing HER2 antigen concentration for 35 min of optimum incubation, exhibiting a linear dependency within (10–110) ng•ml^{-1}.
- A subsequent significant study is an attempt by Li and accomplices, who used an immobilized polycytosine DNA sequence housed in an Au NP matrix as the sensing probe to capture HER2. The response was quantified as the

electrochemical current intensity generated at the electrode surface, corresponding to the reaction between polycytosine DNA phosphate backbone and molybdate. The optimized configuration exhibited a linear response from 1 pg•ml^{-1} to 1 ng•ml^{-1}, having 0.5 pg•ml^{-1} as LOD, ruling out the nonspecific activity entirely among the human IgG, IgA, p53, and CEA. The next crucial attempt is the study of Arya and colleagues, who used thiol-terminated DNA aptamer-engineered interdigitated microelectrodes (IMEs) for HER2 detection, making use of Au electrodes as sensing detectors. The optimized configuration exhibited a significant selectivity in working, detecting the multiple serum proteins within the 1 pM to 100 nM linear range. A more recent attempt by Carvajal and colleagues worked out to make an inkjet printer-configured electrochemical sensor, having the analysis platform comprising an inkjet Au 8-electrode array, a counter electrode, and an inkjet-printed Ag electrode, which has been bleach chlorinated to form an Ag/AgCl quasireference electrode. The working assay was based on the detection of an immunological response, sing the microfluidic device labeled with streptavidin/HRP composite. Designed sensor configuration responded well with 15 min assay time and an LOD of 12 pg•ml^{-1}.

- The above studies illustrate a significant diagnostic accuracy with multifunctional probe designs for HER2 identification. Much has been accomplished due to a high surface area of NMs through which their functionalization potential and interactive sites are greater. It is important to understand that though broad-working mode classifications list all developed configurations in the electrochemical regime, the exact mechanism and probe sensitivity may differ. Thereby, a particular sensor configuration suitable for HER2 detection in advanced stages may not be that much efficient in the initial cycles, whereby the corresponding HER2 expression is well below the desired threshold.

Mucin 1: Attempts to monitor mucin 1 protein expression for possible progress in BC manifestation are described in Table 17. The first notable effort in this direction was made by Zhu and colleagues, who used aptamer–cell–aptamer sandwich architecture to detect MUC1 in MCF-7 BC cells. The developed sandwich-configured biosensor worked well only in the presence of targeted cells. The electrochemical response is generated from HRP labeling on MUC1 aptamer, the subsequent monitoring of electron mediator thionine with an enhanced specificity on doubling the aptamer recognition ability.

- Next in line is the study by Li and associates, who used an electromagnetic approach based on SPR as the MUC1 detection method on MCF-7 cells. The aptamer-functionalized Au nanorods sensed MUC1 within (100–10^5) cells•ml^{-1} range and an LOD of 100 cells•ml^{-1} in 30 min on the whole. The following attempt is the study by Wang and associates, who designed a sandwich-configured electrochemical biosensor working through a polyadenine-aptamer-modified Au electrode and a polyadenine-aptamer-functionalized Au NPs-graphene oxide hybrid for a label-free sensitive MUC1 detection in

MCF-7 BC cells. The sensor detected as low as 8 cells•ml^{-1} with a (10–10^5) cells•ml^{-1} linear range.

- The latest effort toward MUC1 screening is the study from Gupta and associates, who used conducting attributes of a polymer nanocomposite to develop an electrochemical aptasensor. The nanocomposite Au NPs film and graphene oxide-doped PEDOT were developed over the surface of fluorine tin-oxide glass. The configuration enabled MUC1 detection to as low as 0.31 fM with eight times apta-electrode reusability.

CA 15-3: Cancer antigen 15-3 (CA 15-3) is a carbohydrate-containing protein antigen and a member of a mucin protein family. Although the exact role of Ca 15-3 in BC progression is not completely known, it reduces cell–cell interaction alongside inhibiting cell lysis. To date, the major involvement of CA 15-3 protein in BC patients has been from the diagnosis aid, in particular for BC recurrence and metastasis screening during active, therapy resulting from allowing organ specificity.

- The first notable effort toward screening CA 15-3 expression in BC pathogenesis is the study by Zhu and colleagues, who reported a label-free optofluidic ring resonator for a rapid CA 15-3 detection. The optimized configuration functioned well in diluted human serum with a least detection extent of 1 U•ml^{-1} in nearly 30 min. Subsequently, Elakkiya and associates used optics as transducer technology using a cadmium sulfide QD surface capped with cysteamine. The optimal configuration was evaluated in saline and antigen-spiked serum samples and was able to detect 0.002 KU• l^{-1} extent of CA 15-3 with an unchanged response time of 15 min. In furtherance, Marques and accomplices developed the first multiplexed electrochemical immunosensor for a simultaneous CA 15-3 and HER2 screening. The configuration was made using a personalized dual SPCE with electrodeposited Au NPs-modified surfaces on an in situ scale. The electrodes were thereafter coated with monoclonal antihuman CA 15-3 and HER2 Ab. The detection was controlled by voltammetric analysis with 5 U• ml^{-1} LOD.
- Chronological efforts comprise the studies of Ge, Li, and Akter research groups, wherein graphene-based electrochemical immunobiosensors were developed. The attempt by Ge and the group used a nanoporous/graphene hybrid as a platform, having liposomes with encapsulated HRP as labels. A CA 15-3 presence triggered the HRP release from liposome, alongside reducing H_2O_2 with thionine as an electron control agent. The encapsulation significantly aided in the signal amplification facilitating a 5 μU•ml^{-1} as LOD. In the study, Li and colleagues applied graphene to an electrochemical immunosensor in an N-doped graphene sheet configuration. The approach conferred a high conductivity to graphene-engineered electrode, resulting in adequate electron transfer and high sensitivity without any labeling requirement. The optimized configuration revealed an LOD as low as 0.012 U• ml^{-1} within (0.1–20) U•ml^{-1} linear response range. The attempt of Akter and colleagues employed noncovalent functionalized graphene oxide as sensing probes and multiwalled CNT-supported ferritin linkages as labels, both conjugated to CA 15-3 Abs.

The detection of CA 15-3 was facilitated by the amide bond between $–NH_2$ groups of secondary Ab, ferritin, and –COOH groups of multiwalled CNTs, through an enhanced bioelectrocatalytic reduction of H_2O_2, mediated via hydroquinone probe on the functionalized graphene surface.

- The latest effort in CA 15-3 detection is the study of Riberiro and colleagues, who developed an electrochemical biosensor having a synthetic receptor film working through molecular imprinting. The methodology involved CA 15-3 imprinting on a poly(toluidine blue) film following which the assays were performed in buffer and artificial sera, enabling a selective CA 15-3 adsorption onto the MIP film on 30-min incubation. The analysis of calibration plots revealed a linear dependence of CA 15-3 within (0.10–100) U• ml^{-1} range with 0.10 U•ml^{-1} LOD.

miRNA21: Fundamentally, miRNAs are the small, highly preserved noncoding RNA fragments implicated in gene expression regulation. These RNAs are transcribed by RNA polymerases II and III generating precursors that undergo a series of cleavages to form mature microRNA. Figure 22 depicts the molecular functioning of miRNA, wherein binding mediates with 3′-untranslated regions of target mRNA. The bound sequence of miRNA could be oncogenes or tumor suppressor genes, the targeting of which respectively results in tumor arrest and aggravation following the suppression of oncogenes and tumor suppressor genes. The following sections

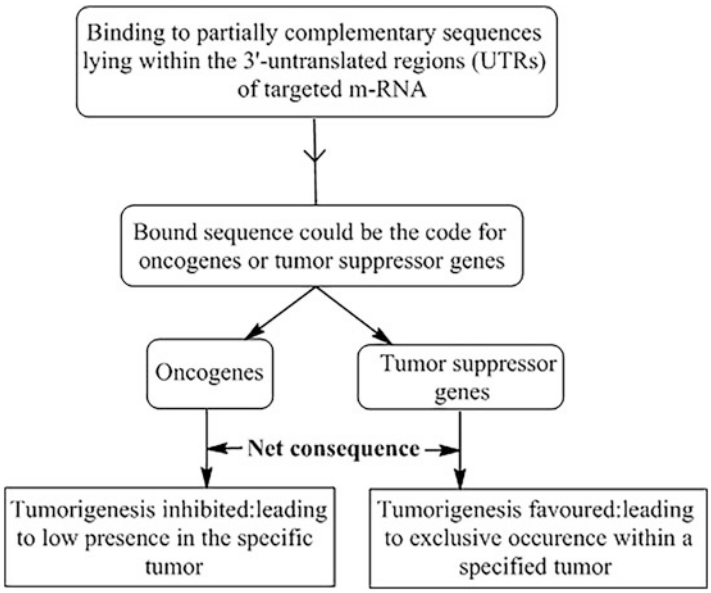


Fig. 22 An overview of the molecular functional controls exercised by miRNA as an antitumor targeting agent, coordinated through the functional activities of oncogenes and tumor suppressor genes

discuss the research attempts toward screening the miRNA 21 expression for ascertaining the BC presence (Table 18).

- The very first attempt in this regard was the study of Kilic and associates, who fabricated an electrochemical biosensor using enzyme-amplified miR21 biosensing from a cell lysate of total RNA. The detection was accomplished through capture probes and covalently attached cell lysates over the pencil-grade graphite electrodes by EDC–NHS coupling chemistry. The optimized configuration operated sensitively with 1 $\mu g \bullet ml^{-1}$ LOD. A subsequent attempt involved a study by Hong and associates, who designed an electrochemical ultrasensitive biosensor for screening the BC-associated miR-21. The probe involved a self-assembled DNA concatamer, wherein a long DNA chain of multiple copies of identical DNA sequences linked end to end, facilitated the miR-21 detection in complex biological samples (enzymes or labels) with 100 aM LOD.
- Next in line is the attempt by Vargas and associates, who fabricated a sensitive amperometric magnetobiosensor for prompt miRNA detection. The detection mechanism involved direct target hybridization through a specified biotinylated DNA probe immobilized on magnetic beads, conjugated with streptavidin. The label comprised a specific DNA–RNA Ab and the bacterial protein A conjugated with an HRP homopolymer for signal amplification. The single-step configuration attained a (1–100) pM linear range and an LOD of 10 aM in a 25 µl sample, devoid of any target miRNA intensification within 30 min. Yet another promising attempt toward miRNA-21 detection is the study of Raffiee-Pour and associates, who used MB as a redox indicator with no label use. The kinetic mode of assays revealed a higher MB stability in binding with miRNA/DNA compared to ss-DNA, generating an LOD of 84.3 fM.

- In furtherance, Li and associates amalgamated a 2-aminopurine probe with a G-quadruplex structure to design a biosensor for overexpressed miR-21 detection from human BC cell lysate without quenchers or enzymes. The optimized configuration comprised two DNA hairpins that considerably elevated the hairpin's fluorescence for a 1.48 pM LOD. Thereby, the fluorescent sensitivity of G-quadruplex-bound 2-aminopurine compensated the requirement of coupling enzymes or reaction quenchers with the probe and simplified the probe interaction. The next attempt in the direction of biosensor-mitigated BC diagnosis is the study of Kangkamano and colleagues, who used a modified electrode to detect miR-21 using a label-free electrochemical biosensor. The designed probe was engineered with pyrrolidinyl peptide nucleic acid/polypyrrole/Ag nanofoam with generated signal exhibiting a direct correlation to the detected miR-21 extents within (0.20 to 10^6) fM. The latest attempt in this regard is the study by Deng and associates, who fabricated an electrochemical biosensor involving a target-induced glucose release from propylamine-engineered mesoporous silica NPs. The optimized configuration used glucose as the signal generating a tag for glucometer readout, wherein labeling and time-consuming repeated washing

steps were bypassed. The sensor worked well with 19 pM LOD. Attempts made toward screening miR-155 as the BC biomarker were relatively fewer and could be traced in Table 18, with two of the three studies using electrochemical transducers.

7 Handheld Devices: Glucometer and Extensions

Glucometer: A glucose meter, more famously a glucometer, is a handheld device for knowing the approximate blood glucose concentration. The alternative version could be a strip of glucose paper dipped into a substance (the concerned medium/environment) after which the developed color is matched with the database of glucose chart. The features comprise the essentials of household blood glucose monitoring (HBGM) for persons suffering from diabetes mellitus or hypoglycemia. The conventional methodology involves extracting a small blood drop via pricking the skin using a lancet before placing it on a disposable test strip that is read by the meter for knowing the blood glucose extent, in $mg \bullet dL^{-1}$ or $mmol \bullet L^{-1}$.

Ever since the 1980s, a major goal of type 1 and type 2 diabetes mellitus treatment had been to attain close to normal blood glucose extents for a longer duration, which is then screened using HBGM. The major incentives of this approach include a reduced occurrence frequency and severity of long-lasting hyperglycemia complications as well as moderating the short-term hypoglycemic life-threatening troubles.

7.1 Working Principle

Multiple configurations of glucometers are known, which invariably rely on the oxidation of glucose to gluconolactone catalyzed via glucose oxidase (GOx), although some of the modules also rely on the oxidation mediated via glucose dehydrogenase (GDH). In general, GDH has a weaker sensitivity over GOx, with a higher susceptibility to interfering reactions involving related moieties.

- The development of glucose biosensors is documented in terms of generation chronology, with the first-generation devices working via colorimetric reactions. These devices are presently in use in the glucose test strips for urinal detection of glucose. Other than GOx, the test kit comprises benzidine derivative that is oxidized to a blue polymer using the H_2O_2 produced amidst oxidation. A significant hurdle herein mandates the periodic development of test strips for the removal of a blood sample, resulting in the requirement of frequent instrumental calibration. The instrument is a handheld device working through a digital model using the transduction of screened glucose excess. Having a changeable ability, present-day glucometers work on the electrochemical principle, with the test strip housing a capillary that is equipped to suck in a reproducible blood quantity. The glucose in sucked blood reacts with GOx or GDH coated on the electrode surface.

- The enzyme is reoxidized in an excess amount of intervening moieties, such as the ferricyanide ion, ferrocene derivative, or osmium bipyridyl complex. The intervening agent hereby undergoes oxidation via electrochemical reaction at the anode surface, generating an equivalent electric current. By Faraday's law, the net charge flowing through an electrode varies directly with the blood glucose amount that has reacted with the enzyme.
- Measurements using the colorimetric method rely on the quantification of the net charge generated by the glucose oxidation over some time while those made using the amperometric method use some meters and quantify the electrical current generated at a specific time instant by the intended glucose reaction. The assessment works through Newton's second law of motion, wherein the progress of glucose reaction is estimated analogous to assessing the rate of change of momentum after an object has been in motion. The analyzed time scales are variable for the colorimetric method but the amperometric mode has the time scales as fixed, although inevitably both methods predict the blood glucose concentration in an analyzed sample.
- A similar principle works in test strips designed to screen the presence of diabetic ketoacidosis. Such test strips use a β-hydroxybutyrate dehydrogenase rather than GOx and have been used to detect and treat certain complications resulting from prolonged hyperglycemia. The sensors for estimating blood alcohol extents have been developed using the same approach, even patented but not yet commercially developed.

7.2 Working Features of a Glucometer

Model-specific working specifications of glucometers regulating their functioning and user acceptability are as discussed next.

- **Size**: In general, the average size of a typical glucometer is equivalent to the hand palm though hospital meters could be the size of remote control. The devices are battery regulated.
- **Test strips**: Test strips inherently provide a sensitive and engineered surface, comprising chemicals that react with the glucose quantity in the blood drop (sample being examined). The strip can be plastic grade, having a small spot impregnated with GOx and other constituents. In general, the strips are fabricated for one-time use although some configurations do employ disks, drums, or cartridges equipped with a consumable amount of being examined material for multiple analyses.
- **Coding**: As test strips vary on batch-to-batch basis, certain models mandate the user to manually enter a code that is written on a particular vial of test strips or on a chip that comes with a test strip. Upon entering the coding or chip into the meter, the device is optimally (through in-built programming) calibrated to that batch of test strips. In case of wrong proceeding, the meter reading may go up to 4 mmol•L^{-1} (72 mg•dL^{-1}), which is quite inaccurate. Such outcomes of

inappropriately coded meters could be serious for patients who are actively managing their diabetes extent and may place them at an enhanced risk of hypoglycemia. Some test strip configurations carry the code information imprinted in the strip while others have a microchip in the vial of strips that can be inserted into the meter. Such design variations minimize the user error likelihood. In some yester designs, a single touch standardizes the test strips around a single code number, wherein the need for a code change is eliminated after it is once set. In some relatively recent models, there is no provision to change the code after it is once set.

- **Volume and time requirement for sample analysis**: Quite often, the typical size of blood drop required for analysis in the differently optimized configurations lies within (0.3–1) μL. The primitive configurations require a large sample quantity (referred to as hanging drop from the fingertip); a smaller volume, however, indeed minimizes the operational fluctuations. The normal time taken to read a test strip ranges from (3–60) s for different model configurations.
- **Alternate site screening**: A smaller drop volume needs pricking of the forearms or other lowly sensitive areas contrary to the fingertips. This testing mode is suited only for stable blood glucose quantities, such as before having meals, amidst fasting, or just before going to bed at night time.
- **Display**: The digital versions of the instrument provide the glucose values in $mg \bullet dL^{-1}$ or $mmol \bullet L^{-1}$. The preferred measurement units vary on a countrywide basis like $mg \bullet dL^{-1}$ is preferred in the United States, France, Japan, Israel, and India. However, in Canada, Australia, and China, $mmol \bullet L^{-1}$ is used. Germany is the only country to date where both units are accepted for measurement. There have been many confusions regarding the measurement units by the instruments, with the patient being misled by the fact that a $mmol \bullet L^{-1}$ reading is a very low reading in mg/dL or the converse. In general, a $mmol \bullet L^{-1}$ value is presented as a decimal point, whereas without a decimal value is in $mg \bullet dL^{-1}$.
- **Glucose versus plasma glucose measurements**: The glucose extent in plasma (one of the blood constituents) is higher than in whole blood, and the difference tends to be ~11% with a normal hematocrit. The understanding of such distinction is significant as household blood glucose meters estimate glucose in whole blood contrary to the plasma extents estimated in most laboratory tests. At present, several meter configurations occupy the market, wherein the result is processed as "plasma equivalent" even though actual measurements pertain to whole blood glucose. The conversion to whole glucose from that of plasma equivalent is facilitated by an inbuilt equation formatted into each glucose meter. Such a facility enables the patients to compare their glucose determinations with the lab test.
- **Provision for yester records**: Many meter configurations at present include a clock that is set by the user for date and time along with a memory for recording past test results. Such a provision is an important step in diabetes care as it aids in keeping a record of management and the gradual shifts (over days and weeks) in blood glucose trends and patterns. In general, most memory chips display an average of recent glucose readings. A common lacuna of all configurations

equipped with clock facility pertains to inappropriate time settings of the clock (due to time changes, static electricity, etc.), whereby the timing of past measurements could be misrepresented, making the management cumbersome.

- **Information transfer**: Several meter configurations, at present, are equipped with sophisticated data-handling ability *vis-à-vis* downloading via cable to a computer having diabetes management software for displaying the test results. Apart from this, some configurations also allow an entry of additional parameters such as insulin dose, the quantity of carbohydrates eaten, and the optimum extent of exercise. Several meters are configured along with insulin injection devices and cellular transmitters. A radio link to an insulin pump allows automatic transfer of glucose measurements to a calculator that assists the wearer in deciding the apt insulin dose.
- **Economy**: Typical expense of a household-used glucose meter is presently high due to the sophisticated needs of test strips used. The 2006 expenses of a glucose meter strip ranged within (0.35–1) $. Very often, manufacturers provide meters at a negligible cost to enable the use of profit-incurring test strips. While type I diabetics may require testing 4–10 times per 24 h (due to insulin modulation extents), type 2 diabetics need a less frequent analysis, particularly in the absence of insulin therapy as a treatment recourse. A recent study funded by the National Health Service, UK, focused on analyzing the cost-effectiveness of self-monitoring of blood glucose and reported significant variations in the customer price not being accountable to the improved working aspects of detecting instruments. The study estimated ~12 million as the spent amount for the 42 million self-monitored glucose tests, which lacked the acceptable accuracy norms. Additionally, accomplishing £23.2 m per annum efficiency savings seemed well within reach, subject to the disinvestment of the National Health Service from the lowly functional technologies compared to the available alternatives (albeit at a higher cost). Inspection revealed some meters as being assembled with counterfeit test strip batches, which substantially contributed to error-prone outcomes.

7.3 Configured Variations

Based on the analyzing ability and the working specifications, the most well-known configurations for glucose meters include hospital glucose meters, noninvasive meters, and continuous glucose monitors. A brief profile of each of these variations is described next.

- **Hospital glucose meters**: Such configurations include special glucose monitors that are now used in multipatient hospitals. These are optimized to provide a more rigorous quality control summary. The data-handling provisions in these systems are optimized to transfer glucose results into electronic medical records and the laboratory computer systems for billing requirements.
- **Noninvasive meters**: Due to a possible risk emanating from continuous skin contact, such glucometer configuration has eluded the users for a long time. The

quest for its exclusive search began in 1975 but has not met any success even to date, with no clinical- or commercial-scale viable product. The maximum progress in this direction relates to a 1999 approval of a sole product for selling, relying on a technique that requires electrical pulling of glucose through intact skin. This product was withdrawn after a short time in light of its poor performance and occasional damage to the user's skin.

- **Continuous glucose meters**: This configuration comprises a disposable sensor positioned beneath the skin, a transmitter connected to the sensor assembly, and a reader for receiving and displaying the measurements. Such sensor types can be used for several days before a possible replacement. These configurations allow real-time measurements and eliminate the need for fingerprick-driven testing of glucose levels. A pressing limitation is an inaccuracy of working as these instruments read the interstitial fluid glucose levels that are much lower than the corresponding blood extents.

7.4 Accuracy Concerns of Glucose Meters

Despite significant technological advances and a periodic review of met accuracy standards for glucose, and ace meters by the International Organization for Standardization (ISO), the accuracy of glucose meters remains a challenging issue. As per ISO15197 Instructions Manual, blood glucose meters must provide results that are within 15% of laboratory standard concentrations above 100 $mg \bullet dL^{-1}$ on a minimal of 95% measurements.

- Disappointingly, several factors could affect the testing accuracy such as proper and upright calibration of meter, optimum temperature and pressure changes for wiping off the strip, size, and quality of blood sample being screened, humidity, aging of test strips, and high levels of certain materials (such as ascorbic acid) in the blood. The model configurations vary in their implicit vulnerability to these factors along with their inbuilt settings to either prevent or warn of the display of the inaccurate results with error messages.
- Recently, an improvised design of conventional Clarke Error Grid (a benchmark standard for analyzing and displaying the reading accuracy), Consensus Error Grid, has been introduced. The older glucometer models still mandate a coding with the used test strip lot failing that the accuracy of reading may tamper on a possible lacking calibration.
- Another pressing issue is the error in the displayed readings for hypoglycemic and hyperglycemic distinctions, wherein the apparent value of instantaneous blood glucose is generally perceived as greater for hypoglycemia than hyperglycemia. This issue has made the meters less useful, with precision and ratio of false-positive and -negative outcomes appearing as primary problems. Such constraints decipher a 15% or low imprecision extent as a limiting factor for hyperglycemic conditions. The working poses a slight distinction in the management of 200 and

260 mg•dL^{-1} extents, wherein an error margin of 15% at a low glucose index manifests a greater ambiguity about glucose management.

- The above reference to imprecision is aggravated by the possible false-positive and -negative outcomes in diabetic and nondiabetic populations. The sufferers of type 1 diabetes in general exhibit a wider range of glucose extents, typically within (40–500) mg•dL^{-1} and corresponding to (50–70) mg•dL^{-1} reading, usually hypoglycemic symptoms are accompanied. So, there is a slight uncertainty about a "true positive" representing reading with accompanied minute harm analogous to its being a false positive. Nevertheless, the occurrence of hypoglycemia (for an unaware state), the associated autonomic failure, and faulty counterregulatory response confer greater reliability at low levels, as desperately urgent in type 1 diabetic patients, which is a comparatively rare possibility with the more common form, the type 2 diabetes.
- Contrary to the above, the error in readings of people not suffering from diabetes pertains to periodic hypoglycemia symptoms, which could also be a higher rate of false positives where the meter is rather inaccurate for a possible hypoglycemia diagnosis. The meter presumably provides occasional usefulness in monitoring severe hypoglycemia (such as congenital hyperinsulinism), corresponding to which average glucose in fastening remains $>$70 mg•dL^{-1}.

Handheld pregnancy detection kit: The first home pregnancy test came into emergence in 1976 when pregnancy tests have become the most common household diagnostic assay. The handheld pregnancy detection provision is a perfect instance of a portable and steadfast biosensor, wherein urine samples are screened for the presence of human chorionic gonadotropin (HCG) using Abs. The choice of HCG as a pregnancy marker pertains to its rapid and consistent rise in early pregnancy, which can be detected in urine. A typical configuration of a handheld pregnancy detection probe is well-equipped to provide women with reliable results just within a few weeks of pregnancy. The working of a pregnancy kit is based on the detection of HCG, which is produced very early in pregnancy by trophoblast cells. On implantation, the placenta begins to develop and generate increasing amounts of HCG, which is screened in the laboratory and home pregnancy kits. For a fundamental understanding, one must be aware of the genesis factor for HCG as a screening agent to ascertain pregnancy, its features, and functions.

7.5 Rationale of Human Chorionic Gonadotropin Measurement for Pregnancy Detection

- The development of the very first biological assay for HCG-driven pregnancy detection can be traced back to 1927 and the combined work of Ascheim and Zondek. In the following 30 years, manifold variations in their procedure have been reported.
- One such modification reported by Tietz in 1965 evaluated the validity in frogs and noticed a 12% positive response in the first week after the missed period. The

extent rose to 58 and 93% in the second and third weeks. On being studied in presumably more sensitive rabbits, the response extents of positivity were 77, 90, and 96%.

- A 1973 optimization attempt by Rees and colleagues demonstrate no serious trouble in devising the tests with a higher accuracy level in the first week itself, after the missed period. Nevertheless, a crucial concern of optimized bioassays pertains to cumbersome and expensive procedures that may consume several days before providing the results. Owing to the convenience and cost compromises, bioassays were superseded by immunoassays in 1960.

7.6 Biochemistry and Functions of Human Chorionic Gonadotropin

- Belonging to the family of glycoprotein hormones, HCG partners with luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyrotropin-stimulating hormone (TSH) as the other family members. Each of these hormones comprises two distinct subunits, the first being the 92 amino acid α-subunit, typically similar in all. However, the second subunit (β) makes up the distinctive features of each hormone. In HCG, the β subunit comprises 145 amino acids, with the first 121 N-terminal amino acids having a common involvement for 80% of the sequence with β-LH. Notably, the C-terminus of β-HCG harbors a 24 amino acid extension, unlike the LH. In general, both subunits perform important biological roles, although the specificity of action is determined by the β subunit. The α subunits encoded by a single gene on chromosome 6 while its β counterpart is encoded by a family of seven genes on chromosome 19 (Chard 1992).
- The bulk of HCG exists as intact (α-β dimer) in the human circulation, with only little extents of free α and β domains. Albeit in urine a major extent of material that participates in immunoassays for HCG is a metabolic fragment of the hormone, commonly known as β-core (Chard 1992). This further comprises two polypeptide chains, derived from the β subunit of HCG, residing in the amino acid sequences 6–40 and 55–92, fused via disulfide bridges.
- The typical β subunit fragment lacks the prominent 30 amino acid carboxy-terminal peptide but does have the constitutional immunological determinants and intact free β subunit. Although significant amounts of β-HCG are located in the urine of pregnant women as well as in some cancer sufferers, it is untraceable in the serum. The rationale for the β-HCG secretion correlates it either via placental circulation after which it is spontaneously excreted by kidneys or anticipate its substantial generator as intracellular degradation of intact HCG β subunit in the renal parenchyma.

7.7 Functions of Human Chorionic Gonadotropin

- Typically, chorionic gonadotropin exhibits several biological actions and functions comparable to those of the pituitary luteinizing hormone. Usually, the trophoblast surrounding the blastocyst commences the HCG secretion in the maternal circulation, normally after a week of conception (implantation stage).
- Increasing extents of HCG in a mother's body confer a stimulus to the corpus luteum (CL, the autotrophic hypothesis). Contrary to this, a normal, nonpregnant menstrual cycle has the CL commencement as involuntary at this stage. Besides, the secretions of estradiol and progesterone are much less, resulting in endometrium loss.
- The CL continues to grow under the HCG influence and onsets with the secretion of increasing steroid quantity. Thus, HCG is the principal signal from an early pregnancy leading to CL rescue, postponed menses, and hence, the maintenance of pregnancy.

7.8 Human Chorionic Gonadotropin in Early Pregnancy

- In general, HCG detection in a mother's physiology can be done 8 days after conception. A titer of ~10 mI, $U \bullet ml^{-1}$ is observed within the serum on the 9th and 10th days, after the follicular rupture. However, with gradual pregnancy development, the HCG count increases by ~50% per day extent. This reaches a saturation extent of ~100,000 mI, $U \bullet ml^{-1}$ by the 10th week. Henceforth, the HCG secretion declines and slowly attains stability with an extent of ~20,000 mI, $U \bullet ml^{-1}$ for the remaining pregnancy duration.
- Apart from being present in maternal serum, HCG can be detected in the urine of pregnant women, with appearance and rise exhibiting a similar variation as that of maternal circulation. From the ninth day post-conception, HCG concentration normally hovers around 0.93 mI, $U \bullet ml^{-1}$ after which the HCG expression steadily increases and becomes maximum post 45 days of conception.
- Repetitive studies have inferred an increment in urinary HCG up to nearly 50%, wherein a significant uniformity is highlighted, irrespective of ethnicities. The levels in early pregnancy (16th day onward following assisted reproductive technology) are significantly lower in women with a higher pre-pregnancy body mass index (BMI), which is believed to be due to the effect of adipose tissue-derived signaling molecules on HCG secretion by the implanting embryo.
- Inaccuracies and inconsistency in HCG concentrations could be due to poor recollection of women's LMP. This is supported by the observations of highly low regular monthly cycles (~32%) with a certainty of LMP date. The reasons attributed to this include early pregnancy bleeding, recent hormonal contraceptive use, or breastfeeding. On computing the HCG concentration through surges in LH that stimulate ovulation, this variability is eliminated.

7.9 Constitution of Workable Kit Configuration

- A handheld pregnancy kit is arguably the most used and successful protein detection device for pregnancy screening. The assay functions via lateral-flow immuno-chromatography. The typical working operation comprises a nitrocellulose (NC) membrane equipped with sample addition and adsorbent pads at its two terminals. A conjugate pad housing the monoclonal anti-HCG Ab bound with the due label (such as Au NP and the dye-doped polystyrene micro/nanosphere) is placed in between the NC membrane and the sample incorporation pad.
- In the immediate surroundings of the adsorbent pad NC membrane terminal, a test line and a control line, carrying antibodies against HCG and MAb, are immobilized over its surface, respectively. On adding the test urine sample to the sample addition pad, the sample moves toward the adsorbent pad via capillary action.
- The working configuration of the probe necessitates the capture of a dye label in both the test and control lines in the presence of HCG. In the absence of this, only the control line would appear as colored. It is worth noting for the readers that the kit provides a qualitative result that is visualized by naked eyes without a transducer involvement. However, lately, it has been possible to obtain a semi-quantitative result by incorporating a reflectance-based reader (Fig. 23).
- Recent attempts in this relation have aimed at the amalgamation of lateral flow immuno-chromatographic assays with electrochemical detection. A significant study by McNeil and accomplices herein reported the impedimetric detection of antigen–antibody interaction that prevailed on the test line. In place of a dye label,

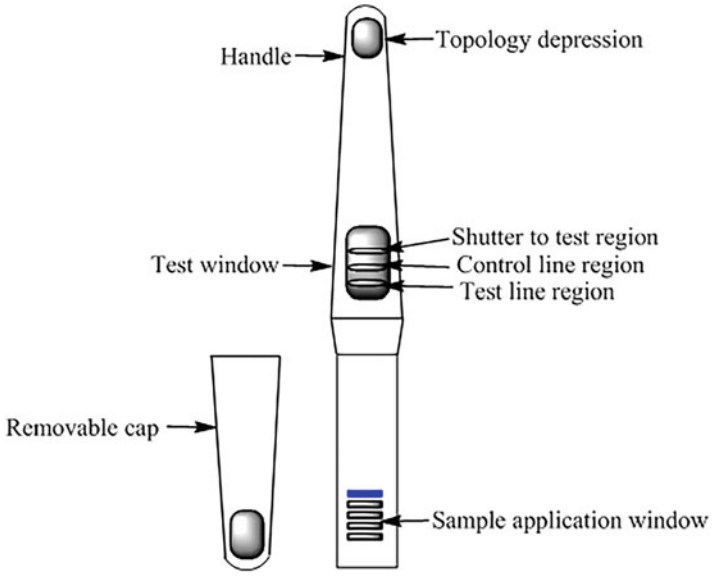


Fig. 23 Working framework of a handheld pregnancy detection kit

urease was used to label the motional phase of MAb. In course of the assay, urea solution was passed over the test line after the capture of MAb–urease conjugate, to get rid of nonspecific residual materials. Detection was ascertained by urease-driven hydrolysis that led to an increase in the pH. To monitor this, a pH-sensitive electrode was positioned over the test line, so that a pH variation caused a breakdown in the polymeric film and effected a detectable change in electrode capacitance.

- In an attempt to use labels other than those of enzyme, Lin and colleagues demonstrated a high-precision assay comprising QD label (CdS@ZnS), wherein electrochemical detection was made by the initial dissolution of QD via acidic functionalization. Analysis revealed the titer of cadmium ions via stripping voltammetric measurement using a disposable screen-printed electrode. Figure 25 depicts the positioning of this electrode below the test line. Investigators emphasized the rationale of the precise measurements using stripping voltammetric assays, making this technology more sensitive over reflectance-based sensing.

7.10 Cautions for Result Interpretation

- Often, it is perceived that pregnancy tests using portable kits are not reliable or are prone to giving negative results before the time of the first period. Nevertheless, a properly and sensitively conducted test with (25–50) $IU \bullet L^{-1}$ sensitivity, a negative result from more than 1 week from the expected missed period can accurately identify the pregnancy.
- Thereby, a fair likelihood prevails of HCG levels in a woman being at the extremely lower end of the normal range leading to its nondetection. Alternatively, it could be possible that conception happened later than expected based on menstrual history. This residual ambiguity can be eliminated by repeating the test after 1 week.
- With current technology, it is quite unlikely to get a positive result in the absence of pregnancy. Still, false-positive results could be obtained, majorly in postmenopausal women. A faint probability mediates such possibilities that are due to low circulating HCG extents in nonpregnant subjects. Several times, it is a coincidence that the screened HCG levels of the test sample do not fall within the instrumental range and are either low or excessively high. In such cases, the first result is indeed not positive. But a repetition of such issues has resulted in the generalization of <5 $IU \bullet l^{-1}$ being confidently stated as negative while those with >25 $IU \bullet l^{-1}$ can be confidently declared as positive. The intermediate values are inevitably a doubtful zone, and the values close to 25 $IU \bullet l^{-1}$ need a retesting at the 2-day interval to assure positivity.
- Several factors have been described at varying time instants as the possible interfering agents in a pregnancy test. Such artifacts in the current generation of test kits could be deciphered from the fact that there is no drug or physiological state due to which well-designed ELISA analysis to be carried over a urine sample could be interfered with. A nonfollower herein is the case of a woman who has

received HCG therapeutically, usually for ovulation induction. This is because the serum half-life of injected HCG follows a multiphase curve with initial rapid stages of 5 and 24 h, followed by a further moderation to 2.3 days. It is not surprising herewith that exogenous HCG may still be detected in urine up to 14 days after the last dose.

- Another untoward possibility pertains to higher HCG expression following intramuscular compared to subcutaneous injection. In such circumstances, the detection of HCG is not unbiased, and therefore, other tests are suggested. Secondly, in case a pregnant woman undergoes an abortion in the first trimester, it may take as long as 60 days for HCG levels to reduce to zero from the comparatively higher values after 7–10 weeks. Yet another highly rare possibility could be the fact that the screened woman may have an HCG-secreting tumor, gestational trophoblastic disease, a gonadal teratoma, or carcinoma with ectopic HCG secretion.
- Above all, it is highly essential to note that pregnancy or a positive test does not assure getting a baby 9 months later. The net loss of pregnancies between conception and term is highly significant. The modern test kit is more likely to result in a negative response for a pregnancy prediction. Usually, the earlier the pregnancy diagnosis, the more likely the abortion chances. Positive tests may even occur in the luteal phase of an otherwise normal cycle, wherein the pregnancy implants but usually aborts before the termination of the expected period. This kind of situation is also referred to as "occult" or more commonly as "biochemical pregnancy."

8 Recent Advances in Biosensor Development for Screening Some Critical Disorders

This section discusses the elect advances of the past 5 years focused on biosensors development of Alzheimer's disease (AD), cystic fibrosis (CF), and tuberculosis (TB). The selection of these diseases has been made based on the alarming adversity affecting healthcare in the recent past. Although this selection is not in its entirety, we believe a thorough understanding of functional principles *vis-a-vis* exclusive pathogenic markers could be crucial toward accomplishing similar advances toward similar fatalities. All these diseases are known for more than three decades now and therefore population affected by them is quite high, owing to which we have chosen these to discuss whether the biosensors equipped with their prompt diagnosis could make a difference in their fatalities or could better the corrective response. Much has been talked about improving the therapeutic efficacy of delivered drugs in these disorders, and realizing which modified routes to prolong the drug therapeutic activities at the desired site is swiftly gaining momentum across the globe. The discussion does not intend to cover all the recent biosensors evidently, and the focus

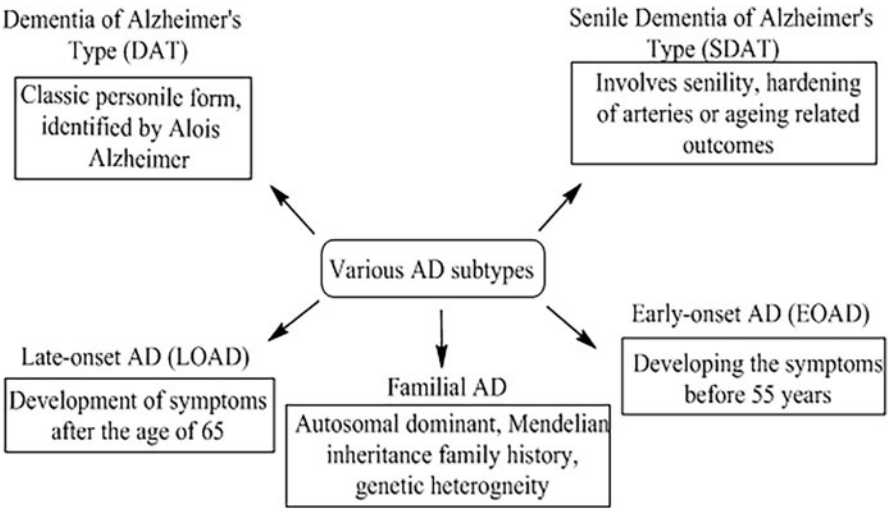


Fig. 24 Characteristics of the various Alzheimer's disease subtypes

is more on the distinct configurations optimized via distinct probe modification including functionalization with NMs.

8.1 Alzheimer's Disease

- Prevailing as the most common aging disorder, AD has been a consistent element of interest for countless clinicians and scientists. Fundamentally possessing a neurodegenerative origin, AD may remain undistinguished by the affected patients and family members from conventional dementia as a part of the clinical syndrome. Even clinicians not having an exclusive specialization rely on default diagnosis for dementia-affected persons despite negative diagnostic tests. Nevertheless, to specialists, AD is an eventual outcome of cognitive decline and is lesser a diagnosis of exclusion but more a recognizable possibility.
- From a histopathological viewpoint, the extracellular amyloid plaques are the first defined signatures along with the intracellular neurofibrillary tangles (90% prevalence as per the post-AD identification by the pathologists). Perhaps, the hallmarks documented and reported more recently include synaptic degeneration, hippocampal neuronal loss, and aneuploidy. As a matter of interest, even non-demented elderly individuals have been screened by pathologists as to having positive AD signatures. This has led to the genesis of preclinical AD terminology that assumes preceding clinical changes by histopathological changes. Owing to this, a longer lifespan of preclinical AD sufferers is likely to result in dementia (the prevalence of histopathological changes). Though studies explore the reliability of this outcome using in vivo plaque and plaque-tangle imaging, to date

consensus considers nondemented elderly cases as equivalent in terms of plaque burdens to those diagnosed clinically with AD.

- A second intriguing aspect of AD molecular biology is it is likely to occur in 95% of persons who are >100 years old. Perhaps, a 2005 study noticed some extent of AD histopathology in all the examined brains of >85-year-old patients (Polvikoski et al. 2006). Owing to this, there has been an anticipation of all long-living individuals to develop AD. Some yester studies established as many as (75–90)% centenarians of have dementia syndrome with (85–95)% meeting the least histopathological criterion. Thereby, it would not be surprising to conclude that centenarians are expected to suffer from AD and presumably comprise an exception if they do not have that.
- Another oblivious concern herein relates to a common onset of age-related cognitive skill revival. Yester reports of neuropsychological origin predict that after adulthood attainment insidious cognitive variations begin to manifest. While such changes express through decades but generally represent a decline from the prior more active and adequate functioning contrary to progressive lateral shifts in cognitive strategies. Though such conditions do not pinpoint an AD possession, it would be a big mislead if one does not consider such outcomes as an indication of the AD continuum. The recent notion of mild cognitive impairment (MCI) resolves these issues to a certain extent, identifying a transitional state between normal cognition and dementia. Over the years of continuing improving molecular characterizations now rate MCI as the manifesting AD in their early recognizable extent. An analogous consideration herein is the age-associated cognitive decline (AACD), which exclusively encompasses "benign" cognitive changes.
- Ultimately, it is now considered a norm to include all dementia syndromes, manifested by plaque and tangle accumulation under the AD loop, leading to classify the various AD subtypes. Figure 24 depicts these subtypes, among which senile dementia of Alzheimer's type (SDAT) includes the individuals who have been diagnosed with selenity, hardening of arteries, or just exhibiting an advanced age (since the 1970s). Late-onset AD (LOAD) includes all those sufferers possessing signs and symptoms post 65 years of age. Early-onset AD (EOAD) loosely includes those sufferers who develop signs or symptoms before 55, 60, or 65 years of age. Here, the exact upper age limit is variably defined. Familial AD includes individuals having an autosomal-dominant family history. Though autosomal-dominant inheritance patterns are rare (even among early-onset cases), the FAD cases prevail with genetic heterogeneity, spanning the mutations in amyloid precursor protein (APP) (located on chromosome 21), presenilin 1 (located on chromosome 14), or presenilin 2 (located on chromosome 1).

8.2 Biomarkers and Biosensor-Mediated Early Detection

- As of now, no reliable peripheral and exclusive biomarkers for AD are known for their discrete identification via histological inspection of the brain amidst autopsy. Inspection using PET using the C11-labeled Pittsburg Compound B (a thioflavin

T derivative that selectively binds amyloid-β) has resulted in contrasting outcomes.

- Senile plaques and NFTs residing inside the diseased brain are the ubiquitous detection markers for AD screening. Recent efforts have improved the understanding of senile plaques, gradually characterized via manifold subtypes, such as diffused, primitive, neuritic, compact core, and cotton wool. Of these, the neuritic plaques have been screened as pathologically most relevant, many times compared with plaque possession, a consensus aspect for AD diagnosis at the time of autopsy. Readers must note here that as per the CREAD guideline (commonly used plaque relying regulation), standardized brain regions are examined through Bielschowsky silver methodology, typically a nonspecific impregnation responding to all plaque morphologies.
- NFT represents the other major hallmark, occasionally referred to as globose and pretangles, although NFT is not semiquantitative in the standard criteria but prevails under the influence of location-specific residence within the brain. A detailed and more rigorous characterization of a typical AD brain using phosphor-Tau Abs and some other closely related lesions comprise neuropil threads (thread similar phospho-Tau accumulations within neuropil of gray and white matters), and dystrophic neuritis (terminal neuritic swellings) residing within the neuritic plaques.
- Other phenotypes characteristically associated with AD include neuronal and dendritic loss, neuropil threads, dystrophic neurites, granulovacuolar degeneration, Hirano bodies, and cerebrovascular amyloid. The synapse loss was described several years back but has been an exclusive finding in recent literature sources. The more commonly noted "synapse loss" is the most specific identifying feature of AD, thanks to the recent increase in the attempts to link the synapse loss with low n-soluble Aβ. Readers must note herein that inspection of synapse via immunohistochemistry or electron microscopy has no role in AD diagnosis at autopsy, irrespective of its significant association with Aβ or the more general, Tau species.
- A serious issue in distinctive AD identification pertains to the distinction between the pathology of AD and that of aging, in particular, among elderly persons. A recent study highlighting this analyzed the clinical diagnosis with the pathological findings, wherein neuropathologists were blinded to clinical data and observed 76% of cognitively intact elderly brains as AD brains. The identification of lesions has been an AD hallmark since its 1907 description. The plaque constituent was more stringently associated with senile dementia, even when AD diagnosis was not known. The senile plaque and NFT lesions undoubtedly remain the associated factors for Ad diagnosis, with several other changes also being known that are comparatively less specific (granulovascular degeneration, Hirano bodies, pathologically noninterpretable synapse loss). The clarity on the spectrum of diseases in different age groups enhanced over the years with lesions assuming more and more significance. In 1985, the Khachaturian criterion made this distinction entirely quantitative, with older patients requiring more lesions to confirm the Ad diagnosis. The association was based on the fact that the cases

having a positive clinical history might have lesions reduced to half. The identification was finally registered by a consortium after several years, with an intent to facilitate research, establish databases, and simplify the diagnosis procedures, wherein plaques were coined as the most significant factors and for the elderly, more plaques were regarded as the confirming factor. This led to the analysis assuming a semiquantitative version, with the responses of "no, possible, probable, or definite." Herein, the "no and possible" criterion could be deciphered with assumptions that either the older brain is more tolerant of pathological lesions or the lesions have nothing to do with etiology. The fact that both possibilities could not be true at the same time emerged as a mathematical certainty.

- Almost at the same stage, the Braak and Braak criteria came into recognition, which relied more on NFT instead of a senile plaque. These guidelines encompassed comparatively more sophisticated neuroanatomy that was designed as being useful even to those having little or no knowledge. Furthermore, the cases considered while framing these guidelines comprised both demented individuals as well as aged controls, whereas the staging was made irrespective of the disease extent. Keeping this in mind, Braak and Braak noticed neurofibrillary degeneration to proceed in a stepwise manner, from the transentorhinal area (neuronal cells in medial temporal lobe bridge, entorhinal cortex, and temporal neocortex) (stages I and II), to limbic regions (stages II–IV), to isocortical regions (stages V and VI). On being compared to clinical data, the majority of stage I–II cases were revealed as free of cognitive impairment while those from stage V–VI exhibited cognitive impairment. Interestingly, the stage III–IV patients remained more or less evenly distributed within dementia-positive and -negative outcomes. This analysis created apprehensions regarding considering the Braak stage I–II subjects as AD positive. Despite such conflicts, the involvement of neurofibrillary pathology along with a significant neurofibrillary pathology and clinical disease correlation, most neuropathologists favor the Braak approach over the CERAd criterion.
- Relatively more recent NIA-Reagan criteria compromise the CERAD (supporting amyloid cascade) and Braak considerations (primacy of phosphorylated Tau), depending on the pathological extent, and divide the cases into "low likelihood," "immediate likelihood," and "high likelihood" of AD.

The following sections describe the major results of select studies focused on early AD diagnosis using biosensor technology collected from PubMed and features from 2016 to date.

- Realizing the significance of amyloid-beta peptide 42 (Aβ42) as a recognition marker of AD manifestation, Li and colleagues designed a reusable biosensor having magnetically sensitive nitrogen-doped graphene (MNG)-modified Au electrode as the detection surface. The Abs of Aβ1-28 configuration is used as the Aβ42-specific biorecognition element, which was conjugated on the MNG surface. The sensing framework could be robustly made using MNP-coated

MNGs, whereby the need for electrode drying could be bypassed, reducing the analysis time and simplifying the overall detection mechanism. The developed configuration exhibited significant reusability with good reproducibility and stability and exhibited a linear correlation within the (5–800) pg•ml^{-1} range. The cut-off extent for Aβ42 was well-resolved with a 5 pg•ml^{-1} LOD. Briefly, the superparamagnetic magnetite (Fe_3O_4) NPs are deposited over nitrogen-doped graphene to form MNG. The MNG surface was labeled with anti-Aβ Abs via sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) cross-linking method to form magnetic immunocarriers (Aβ-MNG). Thereafter, the magnetic immunocarriers were dropped over the Au electrode, leading to their entrapment via placing an external magnet beneath the electrode to facilitate electrochemical Aβ detection. Besides improving the detection efficacy, the optimized configuration also moderated the costs and response time, projecting its diagnostic usefulness (Li et al. 2016).

- Through another significant attempt, Azimzadeh and their team developed an ultrasensitive electrochemical nanobiosensor optimized for miR-137 quantification as a distinct AD biomarker. The designed configuration involved electrochemically reduced graphene oxide (ERGO) and Au nanowires to modify the surface of SPCE after the application of an intercalated label, doxorubicin. The fabrication steps were rigorously analyzed using field emission scanning electron microscopy (FE-SEM), energy-dispersive spectroscopy (EDS), cyclic voltammetry (CV), and EIS. It was found that the sensor worked well within the (5–750) fM linear range with 1.7 fM as LOD. Besides, the sensor also exhibited a significant specificity of working, distinguishing the target oligomers from the nonspecific counterparts (nonspecific targets being miR-21 and miR-155) very well. Inspection of working configuration in human serum revealed its potential significance for prompt feasible and robust AD detection (Azimzadeh et al. 2017).
- Another significant 2017 attempt by Yoo and associates involved the design of an IME sensor for impedimetric Aβ protein detection from the blood. The sensing probe was fabricated through a surface micromachining approach, capable of detecting tens to hundreds of pg•ml^{-1}Aβ contents via medium change from plasma to PBS buffer with signal cancellation and amplification processing (SCAP) configuration. The maintenance of stability amidst medium change was accomplished using a steadfast Ab immobilization method. The probe displayed significant Aβ detection selectivity due to the specific Aβ affinity to the screening Ab at varying concentrations. To synchronize with the optimized functioning, the medium change was optimized concerning optimized absolute impedance change and differentiated impedance variations for real-scale plasma Aβ detection. The output signal processing system of the developed probe was designed for performance optimization and enhancement of signal interaction between recognition and target materials with cancellation and amplification functions for nullifying the parasitic capacitance, error, and noise. The optimized configuration discriminated the Aβ levels between Aβ protein precursor/presenilin 1 (APP/PS1)

transgenic mice and wild-type (normal) mice through screening the Aβ extents in plasma with high sensitivity and reproducibility (Yoo et al. 2017).

- Another notable attempt by Qin and associates designed a label and Ab-free electrochemical biosensor for the exclusive detection of amyloid-beta oligomers (AβO) using an electrically conductive poly(pyrrole-2-carboxylic acid) linking agent and prion protein (PrP^c) receptor. The PrP^c was modified to have an $–NH_2$ group terminal that facilitated a covalent linkage with the –COOH group of PPy. The PPyCOOH (poly(pyrrole-2-carboxylic acid)) was electrochemically deposited on Au substrate and subsequently used for immobilizing PrP^c. To ensure the effectiveness of the conductive linking agent, a nonconductive self-assembled monolayer (SAM) of 3-mercaptopropionic acid (MPA) was layered on Au substrate to immobilize with $–NH_2$-terminated PrP^c. The working principle of optimized configuration was based on the fact that any change in electron transfer through SAM on the electrode surface would be reflected through varied sensor responses. The configuration sensitively detected the Aβ oligomer and developed an insignificant response to the Aβ monomer and fibrils. The working configuration of the sensor was stepwise optimized using EIS and CV, revealing a 10^{-4} pM LOD. To validate the specificity, the designed biosensor estimated AβO levels in cerebrospinal fluid of AD-infected mice, the results of which confirmed an accurate and selective detection at sub-pM extents (Qin et al. 2018).
- In a 2019 study from China, Zhang and associates designed an artificial peptide nanonetwork biosensor to combat its natural contemporary pathological peptide aggregates by mimicking their pathogenic response. Precisely, periphery platelet configuration is capable of secreting Aβ and further induced its cross-linking and aggregation to form a surface peptide nanonetwork. The outcome of such a response was the manifold poly-tyrosine strands that were covalently trapped within the network and served as efficient signal amplifiers through electrochemical oxidation of tyrosine. The method is relatively unique, sensitive, and bears proximity to stress-aggravating Aβ configurations. The designed probe enabled a quantitative distinction of normal and pathological periphery platelet distribution, whereby the AD patients could be effectively discriminated through the screened neurodegenerating platelet functioning. The optimized configuration responded well to clinical blood samples and exhibited a larger dynamic range and a lower LOD compared to ELISA. The signal-amplifying ability of covalently trapped poly-tyrosine strands made the fabricated biosensor significantly better than label-free methods (Zhang et al. 2019a).
- Another benign 2019 attempt by Negahdary and the group fabricated a highly sensitive, eco-friendly, and robust aptasensor for Aβ quantification through electrochemical transduction ability of fern leaf similar to Au nanoassembly. This assembly was synthesized by the investigators through electrodeposition using PEG 6000 as a shape-directive agent. The functioning of this probe was characterized both electrochemically and by FE-SEM. The working configuration involved the detection of Aβ binding on a specific RNA aptamer immobilized on the Au nanoassembly using ferro/ferricyanide as a redox marker. The optimized configuration detected Aβ within the (0.002–1.28) ng•ml^{-1} linear range,

corresponding to an LOD of 0.4 pg•ml^{-1}. The designed configuration functioned well (ruling out any sort of interference), and for assessing the functioning in real samples, human blood serum and artificial cerebrospinal fluid containing Aβ were analyzed (Negahdary and Heli 2019).

- The most recent attempt toward biosensor-mediated early AD diagnosis is a 2020 study by Park and colleagues, who optimized an ultrasensitive and multiplexed detection of $A\beta_{1-42}$ and t-Tau (both prominent AD biomarkers) in biological fluids using a reduced graphene oxide field-effect transistor (gFET). The FET was made by growing a 300 nm SiO_2 layer through thermal oxidation on 4-in p-type Si wafers. The SiO_2 substrates were cleaned (30 min treatment with a 3:1 H_2SO_4 and H_2O_2 solution) and surface activated using high-density –OH functional groups. The cleaned surfaces were then treated with 1%, 3-(ethoxydimethylsilyl)propylamine diluted in ethanol for 1 h to give rise to $–NH_2$ linkage. A GO solution was separately prepared using modified Hummer's method and, after being dispersed in water, was spin-coated on APMES-treated SiO_2 substrate at 500 rpm for 5 s and 3000 rpm for 60 s. This ensured a strong adhesion through electrostatic interaction between the $–NH_2$ group (of APMES SAM) and –COOH group on GO flakes. The GO thin films were reduced through treatment with HCl vapor at 80 °C for 3 h before being patterned via photolithography and reactive ion etching (RIE) to achieve (40 × 80 μm) dimensions. Lastly, the patterned drain and source electrodes were passivated using a stable SU-8 photoresist, resulting in the partial opening of rGO active layer. The optimized configuration functioned well and enabled a linear detection within (10^{-1} to 10^{-5}) pg•ml^{-1} and a femtomolar LOD in biofluids (human plasma and artificial cerebrospinal fluid) as well as PBS. Secondly, the screened biomarkers possessed distinct surface charges in the physiological environment based on isoelectric point, leading to the foundation for achieving a distinct biomarker-specific output signal (Park et al. 2020).

8.3 Cystic Fibrosis

- CF is the most common autosomal-recessive cause of early mortality in Caucasians across the globe, wherein the occurrence frequency is close to 1 among 3000–4000 live births. Nearly 1 in (25–30) Caucasians are the carriers of pathogenic mutation of the CFTR gene. In the United States, as many as 1000 individuals are diagnosed with CF each year. Before the presently followed newborn screening (NBS), the CF diagnosis proceeded either through symptomatic presentation or via family history. In 2004, the Centre for Diseases Control and Prevention (CDC) recommended all states implement the NBS for CF, which resulted in diagnosed extent being enhanced to 2/3rd of all cases. Surveys and databases reveal the NBS-driven early CF diagnosis as the factor behind improved nutritional outcomes that further resulted in improved pulmonary functioning.

- Lung disease is the major source of morbidity and mortality in CF-affected persons, characterized by the most commonly tracked parameter of exhaled air volume in the first second of forced exhalation (FEV_1). Depending on age, gender, race, and height, the FEV_1 can be measured at the earliest in children approaching school age. Children below 6 years are uncertain for reliable spirometry, along with a poor FEV_1 sensitivity for early CF. The noted complementary tests that compensate for these hurdles include infant pulmonary function tests (PFTs), chest imaging, and lung clearance index (LCI), a parameter that identifies abnormalities in the ventilation distribution. Monitoring of such measures by the collaborative efforts of the Australian Respiratory Early Surveillance Team for CF and London CF Collaborative has revealed a nonsuitability of PFT in nearly 25% of 3-month-olds and as much as 50% of 2-year-olds suffering from CF. LCI is abnormal even in 3-month-old children, wherein the early abnormalities are likely to be related to subsequent abnormalities on chest CT scans and are likely to be stronger in abnormality than traditional spirometry in early school years. Thus far, it is highly urgent to search for authentic and reliable early-stage CF detection procedures that could strengthen the treatment efficacy alongside moderating mortality.
- Chronic endobronchial infections have been viciously recognized as contributing factors to CF morbidity and mortality. The recognition of pro-inflammatory organisms, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenza*, *Streptococcus pneumonia*, and *Aspergillus* species, in the first 2 years is associated with worse spirometry in school-going children. *S. aureus* is usually the first respiratory pathogen identified in respiratory secretions of young children suffering from CF, albeit there remains controversy regarding the treatment via antibiotic prophylaxis. In adults infected with CF, *P. aeruginosa* is the most commonly encountered pathogen with its mucoid phenotype playing a decisive role in the manifestation of lung disease. In recent years, *P. aeruginosa* prevalence has been signed in a decline, which could be due to improved control of infection. The methicillin-resistant *S. aureus* is usually associated with deteriorating lung disease with increased mortality. There has been a consistent increment in its existence over the past two decades, similar to that of the non-CF population. Similarly, the *Burkholderia cepacia* complex prevalence has decreased, whereas that of non-tuberculous *Mycobacteria* has increased since 2010. The recent emergence of bacterial detection techniques that do not rely on routine microbial culture assays has identified the diversity and complexity of microbes invading the CF-infected airways.
- Apart from poor nutrition and progressively critical lung diseases, many other manifestations including CFTR dysfunctioning, wherein chronic sinusitis is reported in 30% of all CF patients, lead to poor life quality and require surgical intervention for elimination. Likewise, liver disease is known to affect (15–30)% of CF sufferers with an associated minimal clinical consequence, although, if ignored, it may lead to cirrhosis, along with a liver transplant requirement in severe cases.

- A positive correlation has been noted for CF-related diabetes (CFRD) with progressive aging of patients and gradual destruction of pancreatic islet cells, culminating in insulin deficiency. Lately, CFRD has been associated with more severe lung disease, frequent pulmonary exacerbations, and poor nutritional status. Familiar observations in CF sufferers and their caretakers are depression and anxiety, as a caution of which routine screening is unanimously suggested. The typical presence of depression or anxiety is usually associated with suppressed adherence, enhanced pulmonary exacerbations, and worsened lung disorders. The CF complications are more common in individuals having severe genotypes and pancreatic inactivity. The sufferers with mild cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations are typically pancreatic okay with no demonstrable lung diseases or at least having displayed a multitude of measurable abnormalities. Such individuals may suffer from male infertility or recurrent/chronic pancreatitis.
- The most observed cause of CF to date are the mutations in the gene encoding CFTR protein, an ion channel regulating chloride, bicarbonate, sodium, and fluid fluxes at epithelial surfaces. CF lung disease is identified through impaired ion transport, mucociliary clearance, inflammation, bacterial infection, and airway remodeling, together resulting in bronchiectasis and respiratory failure. CFTR may also cause intrinsic abnormalities in host defense cells, including epithelia, neutrophils, and macrophages.
- Change in FEV_1 has emerged as the most well-known endpoint for demonstrating the clinical efficacy of CF clinical trials. Pulmonary exacerbations also serve as primary clinical efficacy endpoints, but generally require elaborate analysis to demonstrate the treatment impact. The notable secondary measures that have supported approval and clinical use of pulmonary therapies include patient-reported outcomes, weight gain, and bacterial density. Surprisingly, to date, no CF-implicit anti-inflammatory drug has been accorded regulatory approval, whereby knowledge about accepted clinical efficacy measures for these agents is relatively scarce.
- Concerning ionic movement, sweat chloride titer, nasal potential difference (NPD), and intestinal current measurement (ICM) are the major in vivo biomarkers to ascertain the normalcy of CFTR functioning. Of these, sweat chloride is a comparatively easier, portable, and reliable procedure to screen the CFTR operation and discriminate the patients based on minimal, partial, and complete CFTR functions. This method is entirely standardized for clinical applications and is being studied in multiple clinical trials, comprising a formidable mechanism of CF diagnosis. The quantified CFTR functions by the sweat chloride test are ably supported by the altered titers of prominent markers for disease severity, viz., age at the time of diagnosis, pancreatic stability, isolated male fertility, and the typical lung disease severity. Nearly all studies of ivacaftor monotherapy in CF patients (having a gating abnormality) exhibit considerable reductions in sweat chloride. Erstwhile investigations in this reference have reported intermediate effects for ivacaftor in CF sufferers having R117H mutation while moderate consequences were observed in the patients having two copies of F508del CFTR

mutation. In general, the consequences of CFTR modulators on the sweat chloride test remain analogous to the clinical benefits for minimal, partial, and complete CFTR functioning. Though discrete variations in sweat chloride have not been yet directly correlated with FEV1 improvements, available aggregate observations signify a distinctly improved assay performance for detaining the biological activity.

- NPD is a direct measurement source of CFTR functioning in the respiratory epithelium, isolating CFTR activity across the nasal mucosa independent of sodium transport and the functioning of other chloride transporters. In general, the NPD is more difficult to perform than the sweat chloride test and mandates a need for specialized equipment and specific training. Recent interests have standardized the NPD performance and analysis across the United States and Europe, driven through SOPs and centralized coordination and interpretation of trial data. The standardized NPD has been incorporated into small investigator-initiated CFTR, other ion transport modulator trials, and also onto the early-phase trials of CFTR modulators for which regulatory approval needs to be sought. In multicenter trials, NPD assessments exhibited adequate sensitivity to detect dose-dependent ivacaftor bioactivity in patients possessing the G551D CFTR mutation but could not detect the bioactivity of systemic ataluren or lower dose lumacaftor monotherapy in phase 2 and 3 studies conducted on patients having PTC and F508del mutations, respectively. Neither of the attempted interventions could enhance the clinical benefits to a measurable level, inferring the specificity for clinically relevant modulator bioactivity.
- Another assay equipped with the elimination of CFTR functioning is the ICM, exhibiting the distinction of a large dynamic range between CF and non-CF. The typical working comprises a dissection of rectal biopsies that are subsequently analyzed using chambers to monitor the CFTR-dependent ion transport in the ex vivo conditions. Akin to NPD, many illustrious efforts are presently being made to obtain standardized ICM performance and practical suitability, currently in the phase of a universal SOP development by the European centers and US clinical laboratories for centralized data interpretation. An added advantage of ICM is its discrimination of variable extents of CFTR defects, typically based on the characteristic CFTR genotype (nonfunctional, partial, and fully functional) with clinical correlates emerging dependent on CFTR expression. The applicability of an ICM assay is restricted to centers with expertise in electrophysiological measurements. These restrictions have reduced the ICM screening as an early phase CFTR biomarker in molecular diagnosis.

- **Biomarkers of infection**: Markers in this domain pertain to viral and fungal infections, wherein infection by some defined bacterial species (mentioned before) results in CF morbidity and mortality. Most evident infection biomarkers reside in the lower respiratory tract (bronchoalveolar lavage, BAL fluid, sputum) while other sources including cough swabs, oropharyngeal swabs, and nasopharyngeal samples are used in nonexpectant patients. The inconclusive sensitivity

and specificity for lower airway tract infection generally limit their applicability in drug development.

- Frequently analyzed markers for antimicrobial rationale are the bacterial density (determined in cfu•g^{-1} for sputum while in cfu•ml^{-1} for BAL fluid) and the monitored CF pathogens (primary or secondary endpoints). As of now, the chronic *Pseudomonas* infection-ramifying drugs have been approved by the regulatory agencies, working via reduced microbial density as its endpoint. In several trials, variations in FEV1 are noted as the primary endpoint that may not coincide with the decrements in *Pseudomonas* density. Lately though, targeted PCR-facilitated detection (used for viruses) is being improvised upon for an accurate quantitative estimation of CF bacteria. Detection panels for repeatedly infecting bacterial species are being standardized, relying on the prospect that bacterial enzymes or virulence factors may aid in the early detection of resisted responses.

- **Biomarkers of inflammation**: Markers of this category are suspected to play decisive roles in the development of anti-inflammatory drugs that shape the downstream improvements in CF lung disease for disease modification. These markers could be used in early-phase studies to confirm the known action mechanism of drug candidates. The studies made via yester CF clinical trials convey that anti-inflammatory therapies may not induce immediate improvements in pulmonary function and can only moderate the deteriorating lung functioning. The conventional method to monitor CF lung inflammation is through bronchoscopy using BAL. BAL inflammatory markers have been used as clinical endpoints in pathophysiological investigations and clinical trials of inhaled tobramycin as well as recombinant human DNAse. The bottlenecks of using bronchoscopy (recently recommended by the European CF Society Clinical Trial Network for limiting the use to early-phase clinical trials) have rendered blood and sputum as the commonly collected biospecimens for assessing inflammation.
- Spontaneous sputum expectoration is generally confined to screening the more advanced lung disease (in adolescents and adults). Sputum induction (inhalation of hypertonic saline) improves the sample obtained in the sufferers who do not normally expectorate the sputum; the biomarker assessments are generally comparable in both cases. Noted sputum biomarkers for CF are neutrophil elastase (NE), whose activity provides an idea about the bronchiectasis in CF, and predicts a possibility of likelihood future lung deterioration. The NE assessment has been a consistent indicator of treatment response to pulmonary exacerbations, emerging significant to ascertain a future risk too. Elevated BAL NE also serves as a predictive biomarker of impaired lung functioning and bronchiectasis in young children suffering from CF. Other noted sputum biomarkers significant toward CF clinical aspects include calprotectin, myeloperoxidase, high-mobility group box 1, and YKL-40. Though sputum markers have been known for long, recent conflicts in their observations for a possible CF possession make it challenging to rely upon them *vis-a-vis* drug evaluation for shorter periods. The sputum biomarkers

therefore would only be sufficient for their sensitivity to anti-inflammatory effects in longer trials.

- Reliance on systemic inflammatory biomarkers has been a recent upsurge owing to easier standardized, repeatable, age and disease severity-dependence moderate blood measurements. Systemic inflammation may also link pulmonary and non-pulmonary CF morbidities, although the availability of data linking systemic inflammation with clinical outcomes in CF is scarce. A considerable variation in circulating biomarkers (contrary to sputum biomarkers) of inflammation after exacerbation treatment is a vital inference of a higher sensitivity of systemic inflammatory signatures, highlighting their usefulness in short-term interventional studies aimed at mitigating exacerbations. For example, a multicenter exacerbation study noted suppressed serum CRP, amyloid A, and calprotectin expressions in response to azithromycin treatment during a CF interventional trial. The reductions correlated well with improvised lung functioning and weight gain, suggesting the changes to be associated with clinically meaningful outcomes. Other potential systemic biomarkers relating clinical status to CF outcomes comprise neutrophil elastase antiprotease complexes, several cytokines such as IL-6, IgG, and circulating mononuclear cell RNA transcripts. Readers are suggested to have a look at more focused literature sources to know about the further possibilities of improving the biomarker-predictive CF intensity and the possible future challenges. The following points discuss the salient biosensor mediated earlier and faster CF diagnosis of the recent past.
- In a noted attempt in 2015, the study by Toren and associates proposed a microtoroid surface (as a biosensor) for screening the early-stage bacterial mutations. The specific context referred to herein was for screening the DNA alterations of *Pseudomonas aeruginosa*, the major CF pathogen that suffers from a poor prognosis through manifested mutations in course of virulence and drug-resistance development. The optimized configuration involved the fabrication of high-quality factors microtoroids, after which their surfaces were coated with 3-aminopropyl triethoxysilane (APTES)/trimethyl methoxy silane (TMMS)-mixed silane solution. Subsequently, the engineered toroid surfaces were again coated with EDC/NHS linkages to facilitate the covalent conjugation of DNA probes. Nonspecific interactions were prevented through ethanolamine capping while the homogenous functionalization of the toroid surface was inferred from confocal studies. The bacterial DNA hybridization at each step was observed as dependent on probe length, where a significant response of nearly 22 pm was obtained in response to the complementary strand of mutated *P. aeruginosa* DNA, while that toward the mismatched strand was substantially low and late (~5 pm). The LOD for the complementary strand was 2.32 nM, whereas no significant response toward the noncomplementary strand was developed. Thereby, it was validated that engineered microtoroid surfaces were capable of distinguishing DNA alterations and CF-specific *Pseudomonas* recognition (Toren et al. 2015).
- In a 2018 study reported from Saudi Arabia, Eissa and colleagues proposed a multiplexed biosensor for the detection of hyper immunoglobulin E syndromes (HIES), the rare immunodeficiency disorder. The biosensor working domain

spanned a simultaneous detection of signal transducer and activator of transcription 3 (STAT3), dedicator of cytokinesis 8 (DOCK8), and phosphoglucomutase 3 (PGM3) proteins. The sensing probe was constructed on carbon array electrodes that were initially modified through electrodeposition of Au NPs. The array electrodes were thereafter used to immobilize specific Abs for the three proteins after the electrode functionalization with cysteamine/glutaraldehyde linkers. The sensor enabled simultaneous detection of DOCK8, PGM3, and STAT3 proteins with the respective limits of 3.1, 2.2, and 3.5 pg•ml^{-1}. The sensing exhibited good selectivity and sensitivity toward other proteins (along with DOCK8, PGM3, and STAT3), that is, CFTR and Duchenne muscular dystrophy (DMD). The sensor also displayed a distinctive HIES identification from human serum. So, although the designed configuration was not aimed toward screening CF, the identification of CFTR protein attributes to its ability for efficient screening of CF (Eissa et al. 2018b).

- Another attempt from a similar research group demonstrated a disposable carbon nanofiber (CNF)-based electrochemical immunosensor for simultaneous detection of survival motor neuron 1 (SMN1), CFTR, and DMD proteins. The intent behind choosing these analytes was to accomplish a point-of-care detection mechanism for identifying the specific biomarkers of spinal muscular atrophy (SMA), CF, and DMD, all hereditary disorders possessing rising morbidity and mortality. To design the biosensor, initially, the CNF-modified array electrodes were functionalized by the electroreduction of a carboxyphenyl diazonium salt. Thereafter, the sensing probe was designed through a covalent immobilization of three Abs on the working electrodes via EDC/NHS chemistry. The optimized configuration enabled simultaneous detection of CFTR, DMD, and SMN1 with high sensitivity and 0.9, 0.7, and 0.74 pg•ml^{-1} LOD. Apart from this, the sensor also enabled a high recovery percentage on being applied to spiked whole blood samples. Thus, the voltammetric immunosensor configuration provided a cost-effective, easy-to-use, rapid and high-throughput SMA, CF, and DMD detection using merely a few blood drops (Eissa et al. 2018a).
- A second attempt from the Toren research group is a 2018 study that featured in *ACS Sensors* and aimed at the early detection of pathogens or their virulence factors in complex media, suspecting its key role in the early diagnosis and treatment of multiple diseases. The working configuration enabled a nanomolar-selective Exotoxin A, a virulence factor secreted by *Pseudomonas aeruginosa* in the sputum of CF patients. The study described an account of preliminary investigation toward the feasibility evaluation of optical sensing of *P. aeruginosa* in diluted artificial sputum, which mimicked the CF respiratory environment. The probe design enabled a high-throughput screening by exploiting anti-Exotoxin A-conjugated microtoroidal optical resonators. The optimized surface engineering enabled an effective biointerface toward a highly selective Exotoxin A detection in the complex media using monoclonal anti-Exotoxin A-functionalized microtoroids. The engineered surface was screened as highly resistive to other sputum constituents and enabled exclusive Exotoxin A

identification with reproducible measurements, with an LOD of 2.45 nM (Toren et al. 2018).

- A recent attempt from 2020 is a study by Ghaderinezhad and his group, who fabricated a paper-based sensor for quantitative screening of sodium, potassium, calcium, chloride, and nitrite in the urine. The designed sensor configuration was quantified using a smartphone-enabled platform. The probe comprised of a fluorescent-sensitive platform for Na^+, K^+, and Ca^{+2} detection and a colorimetric method for Cl^- and NO_2^- detection. To make the biosensor, the reagents were added to a paper matrix after which the sample was deposited. This was succeeded by reading the fluorescent and color intensities for quantifying the target ion concentration. The application screening of the optimized configuration enabled the Na^+, K^+, and Ca^{+2} estimation in their physiological extents, Cl^- from 50 to 300 mM and NO_2^- from 0.05 to 2 mM in the artificial urine (Ghaderinezhad et al. 2020).

8.4 Tuberculosis

- One of the most dreaded life-threatening disorders, tuberculosis (TN) is caused by *Mycobacterium tuberculosis* (Mtb) and is transmitted by aerosols released from the upper respiratory tract of an infected person to a healthy counterpart. As per the WHO 2016 global TB report, 10.4 million TB cases were reported across the globe in 2015, of which 1.8 million died in the same year. More startlingly, as much as one-third of the world's population is presently infected with Mtb bacilli. It is a further concerning aspect that despite registering a 22% decline in TB deaths during 2000–2015 the disease remains well-settled among the top 10 leading causes of death across the world.
- The exclusive traits of Gram-negative bacillus, Mtb, make TB one of the major prevalent diseases at present. TB can remain in a dormant state for years without expressing symptoms or getting spread to other subjects, but immediately after a host-immune system is weakened, the bacteria become active and exclusively affects the lungs and other body parts.
- The TB sufferers are further aggravated owing to the immune system affecting illnesses that affects the immune system, such as HIV, whose condition is highly precarious in many developing and underdeveloped economies of the world. The bacilli exist in the lungs in the instant just after entering a host's body, when they are contained by the host's immune system, resulting in moderation and latency of infection, without noticeable symptoms. That's why early diagnosis and treatment of latent TB infection is a pivotal step in TB cure and is a decisive factor for preventing the dissemination to healthy individuals.
- The major obstacles to successful field detection and treatment of TB are spearheaded by the omnipresence of pathogens and diseases across different continents, thereby complicating the management and eradication programs. Interestingly, nearly 98% of reported cases are from developing countries,

resulting in TB being coined as a disease of poverty, wherein as much as two-thirds of infections are reported within the (15–59) age group, typical adulthood.

- The next curtailing factor is the high cost of first-line anti-TB drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) that restricts their usage and aggravates the frequent resistance episodes. This factor has been the limiting cause of inadequate progress in emerging TB treatments and has vitally affected countries like India and others.
- The resistance episodes in Mtb toward the major first-line chemotherapeutic agents have marred the treatment of TB cases. The last but not least issue relates to a major extent of date of diagnosis procedures as being immunoassay-driven, resulting in reduced specificity. It is worth mentioning here that Mtb shares antigens with several other Mycobacterium species, owing to which the incidence of false-positive testing is noted in as much as 35% of active sufferers. Besides immunoassays, several other techniques such as flow cytometry, radiometric detection, and latex agglutination are also used but all suffer from one or more disadvantages about time-consuming detection procedure, skilled manpower requirement, nonreproducibility of measurements, costly probe configuration, etc.
- **Current diagnosis**: Present-stage TB diagnosis is done via immunological-cum-microbiological and genotypic tests. The following sections discuss the major characteristics of these techniques with a focus on their inadequacies.
- **Immunological-cum-microbiological tests**: The different configurations of these tests are available for TB diagnosis, mentioned next in chronological order:
  - **Smear microscopy**: Microscopy-based smear tests are very rapid, inexpensive, simple, and relatively easy-to-perform procedures for the detection of acid-fast bacterium such as Mtb. Usually, the Ziehl–Neelsen staining is employed needing at least 10^4 bacteria per ml sputum. The fluorescence microscope using auramine-rhodamine staining is comparatively more sensitive despite being expensive, owing to its necessity of a fluorescence microscope. A higher sensitivity of this method is attributed to the feasible examination suitability of slides at lower magnification. This method processes results within hours.
  - **Immunological assays (latex agglutination, ELISA, and Mantoux tests)**: These tests are based on the typical binding of serum antibodies to Mtb antigen. For instance, in latex agglutination tests, the latex (as polystyrene) beads are functionalized with antigens extracted from a pathogenic Mtb, which are subsequently reacted with serum samples. The completion of the reaction results in coagulation of latex beads demarcating a positive response. These tests do not have a high sensitivity, owing to which these often meet failure whenever the bacterial load is lower than a threshold. Furthermore, the presence of antibodies in the serum could also interfere with the analysis and aggravate the error-prone inferences.
  - **Radiometric detection test**: This test estimates the Mtb metabolic activity through radiological means. For instance, the *Mycobacterium* species are well known to generate CO_2 from the carbon sources like glycerol or acetate. The

important selective criterion concerning Mtb herein pertains to its inability of generating CO_2 from glucose. Using this selective CO_2-generating characteristic, the Mtb capability in generating $14CO_2$ from 14C-U-glycerol or 14C-U-acetate but not from 14C-U-glucose is estimated. This method draws very low responses from developing countries owing to the technical complexity.

- **Flow cytometry test**: Over the past few years, this technique has swiftly witnessed an increment in the detection of TB cases. The method herein exploits the ability of viable Mtb to absorb fluorescein diacetate (FDA) and hydrolyze it into fluorescein, which on being accumulated in the metabolically active bacterium could be viably detected. The reproducibility of this method is high, along with null active mycobacterial cell division. The technique, however, desperately needs a sophisticated infrastructure and skilled manpower, which are seldom accomplished in developing countries.
- **Cultivation detection tests (like BACTEC MGIT 960 systems)**: In these techniques, biological samples are selectively cultured on solid media to detect and quantify Mtb. Though operationally quite accurate, a (9–42)-day growth period is required by this method. The method also requires a laboratory backup, which complicates applications under field conditions in developing countries.
- **Genotypic tests**: DNA-based techniques are rapidly assuming significance, in particular for the Mtb-resistant stain detection. These tests mandate a laboratory-scale optimization and therefore do not represent an apt option. The following are the most important variations of these tests:
  1. **Assays based on polymerase chain reaction**: Of the well-documented and studied molecular assays, PCR-mediated Mtb screening relies on the recognition of specific gene sequences (from the mixture of all host genes

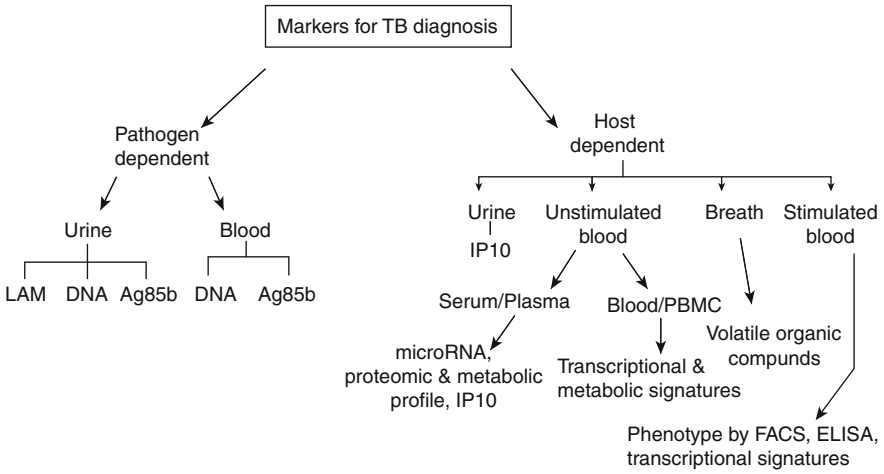


Fig. 25 Diversity of TB diagnosis biomarkers. The current regime has more reliability for pathogen-dependent screening as the detection is exact and prompt

fragments) using the complimentary primers. The PCR-based assays are the most sensitive techniques for screening the codons responsible for Mtb resistance. The DNA fragments amplified by PCR protocol [better termed as restriction fragment length polymorphisms (RFLPs)] are thereafter analyzed using electrophoresis or hybridization-based assays.

The following are the brief operational steps of these techniques:

2. **Electrophoresis**: This analytical technique works based on charge-modulated electrophoretic mobility of the mutated Mtb fragments, especially those of resistant strains. The analysis is made via comparison of PCR-amplified DNA with the electrophoretic mobility of wild/reference Mtb DNA to screen the resistant genotype. In the past few years, a heteroduplex assay has also been reported for the identification of single-base mismatched strands. The strands are isolated from the null mismatched containing strands using the confirmatory gel electrophoresis assay.
3. **Hybridization-based methods**: These methods are based on the hybridization of clinical DNA fragments with those of complementary DNA. The concomitant binding is thereafter compared with the outcomes of wild/reference Mtb DNA. The binding of clinical DNA can be ascertained via ELISA readers in case of the significant homologous complementary strand. The hybridization could be accomplished with the help of strips, microtiter plates, and microarrays.

– **Real-time polymerase chain reaction**: It is feasible to visualize the increasing concentration of a DNA strand in real time using fluorescently labeled DNA strands. Several fluorescent labels are applied in an adverse range of real-time PCR techniques, such as TaqMan probes, Beacons, and FRET probes.
– Exclusive demerits of PCR tests comprise relatively high equipment and reagent costs. The method also mandates the requirement of skilled personnel along with dedicated pre- and post-PCR rooms to keep the contamination at bay. Furthermore, the technological dependence and sample preparation complexity make these procedures inappropriate in resource-poor economies.

8.5 Biomarkers for Tuberculosis Detection

- Figure 25 distinguishes the pathogen and host-based biomarkers, primed for TB diagnosis. From a pathogenic perspective, Mtb products could be aptly distinguished in the blood, sputum, or urine. The blood and urinal detections can be made with a higher sensitivity contrary to that of Mtb culture from the analogous biological fluid. The Mtb cell wall constituent, lipoarabinomannan (LAM), is reported as a discrete TB biomarker in light of the low sensitivity of the conventional urinal analysis.
- Although well below par the conventional standards recommended to date, the Mtb DNA and LAM detection in the urinal samples of HIV-infected patients could be a decisive approach for the detection of advanced TB with low CD4 T-cell counts. Of note, Mtb Ag85 complex is a (30–32) kD family of three

proteins, namely, Ag85A, Ag85B, and Ag85C, characterized by the mycolyl transferase-mediated coupling of mycolic acids to arabinogalactan of the *Mycobacterium* cell wall and in the biological formation of cord factor. Multiple studies pinpoint the significance of Ag85 (in blood and urine) as an Mtb detection marker.

- Among the host biomarkers, several non-sputum-based assays for active TB screening are validated using serum, plasma, urine, or stimulated or unstimulated blood. In light of serum or plasma products, Mtb-specific Ab detection is not an accurate diagnostic method due to a heterogeneous Mtb response pattern. Apart from this, the WHO guidelines also voiced against the use of these tests for active TB diagnosis. Studies on serum microRNAs have been reported with variable success for active TB diagnosis in drug-sensitive and drug-resistant subjects.
- A broad range of potential transcriptional biomarkers have also been reported, including neutrophil-driven interferon (IFN)-inducible gene profiles comprising both type 2 IFNγ and type I (IFNαβ). IFN signaling represents a significant TB signature traceable in peripheral blood from pulmonary TB patients. Such findings have been complemented in the analysis of other populations and in several other studies where the manifestation of TB could be distinguished from other respiratory infections and inflammatory diseases. Correlations with IFN expression have inferred enhanced activity for the diseased state, which decreased for the treatment state.
- Diagnostic indications of a TB distinction from other diseases and LTBI were noticed in children from South Africa, Malawi, and Kenya. Disappointingly, a persistent challenge in the detection of new childhood TB pertains to missing references due to a cumbersome microbiological diagnosis of active disease. Analysis complexity and expensive operational requirements (related to transcriptional profiles) make the diagnosis difficult and urgently require the development of easier technologies.
- IFNγ-inducible protein 10 (IP10) has been studied as being enhanced in the unstimulated plasma of children and adults having active TB. This has been screened using different methodologies based on lateral flow assays using the interference-free, fluorescent upconverting phosphor extents. Of significant interest is the fact that IP10 could also be detected in the urine of adult patients. A study conducted on Ugandan children infected with TB revealed decreased IP10 expressions in response to efficacious therapy.
- Potential breakthroughs in TB diagnosis have also been enabled by the advanced mode functioning of multiparametric flow cytometry, allowing simultaneous screening of manifold immune functions in single cells, such as cytokine generation and memory status. Polyfunctional T-cells are capable of generating more than one cytokine at the same instant and are the active components of the immune response toward different pathogenic species. The T-cells producing IFNγ, TNFα, and IL12 are associated with guarding immune responses in HIV nonprogressing subjects. Studies focused on analyzing the role of polyfunctional T-cells in TB do not decipher a homogenous conclusive outlook. Active TB has been linked either with monofunctional TNFα-$CD4^+$ T-cells or double-functional

$IFN\gamma^+$ $TNF\alpha^+$ $CD4^+$ T-cells. Contrary to this, the activation and memory states of Mtb-specific T-cells are generally more in agreement, even when comparing patient populations at different sites.

- While the effector T-cells grow amidst Mtb replication, the memory cells regulate the arrest and elimination of Mtb infection. It is pertinent to mention here that active TB infection is profusely estimated by suppressed CD27 surface expression over the circulating Mtb-antigen-stimulated $CD4^+$ T-cells. More recently, an erstwhile T-cell activation marker, (TAM-TB) assay has been demonstrated for active TB recognition in children. This assay relied on the stoichiometric variations of median fluorescence intensity of all $CD4^+$ and $CD27^+$ T-cells to those of Mtb-explicit $CD4^+CD27^+$ T-cells (CD27 MF1 ratio). This marker has been evaluated in adults from a low TB-endemic country, where it successfully distinguished the various stages of a TB infection.
- A breakthrough study based on blood analysis showed the expression of immune activation markers, CD38, HLA-DR, and proliferation marker, Ki-67 on Mtb-specific $CD4^+$ T-cells, involved with Mtb load. It was noticed that modulation of these markers adequately distinguished active TB cases from those of LTBI with 100% specificity and over 96% specificity. These markers also classified the individuals who had completed TB therapy, indicating a correlation with the decrease in mycobacterial load after treatment. Of eminence is the recent finding of T-cell activation as an immune correlate of risk for TB development in BCG-vaccinated infants.
- Nontargeted methods to screen and identify novel markers for TB sufferers are primed on transcriptomic, proteomic, and metabolomics hypotheses. A noted attempt herein analyzed the transcriptomic and metabolic contour of patients infected with two distinct Mtb lineages (Maf and Mtb), before and after anti-TB therapy. The main aim of the investigation was to identify and enumerate the distinctions in host factors and biological events associated with diseased pathology and the corresponding explicit treatment response. It was noticed that peripheral blood gene expressions were not significantly distinct between the Maf- and Mtb-infected patients at the pretreated stage. However, the extents considerably differed at the post-treatment stage. On the whole, the investigation summarized that the pre- and post-treatment genotypes were exclusive functions of the characteristic immune response.
- It is urgent to mention herein that the upstream regulator hepatocyte nuclear factor 4-α regulated ~15% of distinctively expressed genes in the post-treatment groups. Surprisingly, the serum metabolic profiles of Maf and Mtb strain-infected sufferers remained unchanged both at pre- and post-treatment stages. Subsequently, the advent of analytical tools like mass spectrometry or protein chip technology enabled the distinctive analysis of the proteomic profile of TB sufferers and healthy individuals. The noted host markers that distinguished the TB sufferers from those suffering from erstwhile infections and inflammatory disorders included transthyretin, CRP, and neopterin. Interestingly, the sputum analysis also distinguished the sufferers via analyzing the proteomic profiles based on smear-positive and -negative TB patients contrary to those of control models.

- The biomarkers specific for active pulmonary TB may prevail in the volatile organic compounds (VOCs) in the breath, both from the infecting organism and the infected host. These signatures could be the outcomes of concomitant oxidative stress. For a definitive screening, a breath test for the presence of VOCs could be done, wherein potential biomarkers of active pulmonary TB could be ascertained with ~85% accuracy in symptomatic high-risk individuals. The presence of VOCs in the picomolar range complicates their screening, typically remaining out of the measurable extents of the majority of conventionally used instruments.

8.6 Biosensor-Mediated Early Tuberculosis Diagnosis

Knowing that 98% of all TB cases prevail in developing countries, where access to specialized and advanced facilities in a laboratory setup is scarce, there is a dire necessity to develop alternative, simpler, and low-cost techniques for TB diagnosis. The varying sensing and transduction modes of a biosensor facilitate prompt detection of these responses through simplistic and efficient measurement of optical, thermal, and electrical signals. Major advances in biosensors for a faster TB diagnosis are as follows:

- **Technical advantage**: Biosensors empower a high level of device and capture, followed by integration achieved through single-step detection.
- **Quick response**: The typical response time of a biosensor analysis is within the range of a few minutes in most configurations, together enabling rapid and significant measurement control.
- **Ease of use**: Many designed and optimized biosensor configurations are equipped with user-friendly interfaces for connection with advanced instrumentation.

The following points discuss the most recent biosensor studies aimed at a faster and more efficient TB diagnosis. The salient aspects of performance analysis aspects are briefed.

- A recent attempt focusing on the practical demerits (longer detection time than the conventional approaches for MTb detection) is the 2020 study by Zhang and associates. The investigation reported an electrochemical biosensor using a 16S rDNA fragment of Mtb, H37Ra as the target marker. Peptide nucleic acid (PNA) was used as a capture probe while 2D Ti_3C_2MXenes (having high conductance) were used for signal transduction. Amidst the assay development, the PNA and 16S rDNA-specific fragment was hybridized on the substrate of the PNA-Au NPs network electrode, after which the target fragments were directly conjugated with Ti_3C_2MXenes. This linkage was stabilized by the strong interactions between zirconium cross-linked Ti_3C_2MXenes and the phosphate moieties of target fragments, which bridged the gaps of interrupted Au NPs in the nanogap network electrode, giving rise to a conductive passage to monitor the conductance

variation across the electrodes. The conductance variation was monitored for Mtb screening, which was completed in 2 h with an LOD of 20 cfu•ml^{-1}. The PNA used in the analysis was an unnatural DNA analog, comprising repeating neutral, N-(2-aminoethyl) glycine functionality, linked together via peptide bonds and having nucleotide bases. The as-designed sensor configuration functioned well and enabled the Mtb detection in 40 stimulated sputum samples. Thereby, this study explained the success of the shift from PNA to highly Ti3C2MXenes, across the nanogap network electrodes, enabling the detection using a sharp conductance change (Zhang et al. 2020).

- Another rigorous 2020 attempt from Pelaez and colleagues reported a portable, user-friendly, and low-cost biosensor working in a label-free manner and using SPR sensitivity. The configuration was a direct immunoassay for spontaneous detection and quantification of the heat shock protein X (HspX), a standard biomarker of Mtb, prevailing in pretreated sputum samples. The sensing mechanism relies on highly specific MAbs that were previously immobilized on the plasmonic sensor surface. The optimized configuration enabled direct detection of Hsp without amplification, with a 0.63 ng•ml^{-1} LOD and 2.12 ng•ml^{-1} as the limit of quantification (LOQ). Also, the inspection of pretreated sputum samples revealed significant differences in the Hsp patient concentration, for those infected with TB, (116–175) ng•ml^{-1} concentration extents compared to below LOQ for the uninfected subjects (Pelaez et al. 2020).
- A highly significant attempt by Gupta and colleagues used a magnetic biosensor relying on giant magnetoresistance (GMR) for on-field TB detection through assessing an Mtb-specific protein, ESAT-6 (early secretory antigenic target). The specific reason to choose ESAT-6 as the MTB detection protein was its secretion at the initial infection stage besides a prominence in progressive TB infection. The investigators opined that due to a molecular weight of 6 kDa, ESAT-6 is a small protein that can bind to toll-like receptor-2 (TLR-2) directly and also exhibits an inhibiting capability toward the downstream transduction of signaling events. Furthermore, ESAT-6 can form a heterodimer with culture filtrate protein (CFP-10), another MTB-secreted antigenic protein, having a molecular weight of 10 kDa. The portable configuration served as a highly sensitive diagnostic tool and detects MTB with a low turnaround time and a pg•ml^{-1} ranged LOD. The use of DARPins (designed ankyrin repeat proteins) in the optimized configuration confers a high specificity and aids in prompt detection, thereby enabling early treatment onset with reduced mortality. The comparison with conventional and Au NPs-assisted ELISA inferred the developed configuration as more sensitive. Utility analysis revealed a likelihood of sensitivity improvement through variation in the structural composition and size of MNPs, with the larger-sized NPs (40 nm radius) exhibiting higher signal strengths for low concentrations compared to small-sized NP (5 nm radius). Besides, there is the feasibility of real integration of GMR biosensors with several other standardized procedures; it was found that this technique could be sued for the detection of malignant pathogens such as HIV and drug-resistant mutants (Gupta and Kakkar 2019).

- Another significant attempt by the same group (Zhang et al, the first being the discussed 2020 investigation) illustrated the design of an electrochemical biosensor for screening of MTB reference strain, H37Rv. Optimized configuration used H37Rv aptamer (as recognition probe) and oligonucleotides engineered using Au NPs. Detection was made via monitoring the frequency variations, conferred by Au NPs conjugated with DNA, in the presence of H37Rv, with the aid of a multi-channel series piezoelectric quartz-crystal (MSPQC) system. Three oligonucleotides were engineered with Au NPs and were hybridized with 37 nucleotides comprising H37Rv aptamer, which was immobilized over the Au electrode using Au–S linkages. A conductive layer was henceforth generated by the sequential hybridization of the aptamer with the Au NPs-functionalized DNA segments. The binding of the pathogenic (Mtb) strain to the aptamer took place in the presence of H37Rv and resulted in the detachment of Au NPs-conjugated DNA from the electrode surface. The conductive film was thereafter replaced by an insulating aptamer-conjugated bacterial complex. The discrete variations were recorded by the MSPQC system. The optimized configuration facilitated a rapid, implicit, and sensitive detection and provided the response in 2 h with a 100 CFU•ml^{-1} LOD (Zhang et al. 2019b).
- Another elegant study of 2019 itself was reported by Bai and colleagues from China, wherein detection of IS6110 MTB fragment was made using novel sensing mechanisms of an electrochemical DNA biosensor. The sensor probe comprised of a nanohybrid of Au NPs-decorated fullerene NPs/nitrogen-doped graphene nanosheet, which directly served as a new signal tag. This probe generated a signal response without additional redox molecules and was thereafter labeled with signal probes to form a tracer label for accomplishing signal amplification. In an attempt to further improve the diagnosis sensitivity, biotin–avidin conjugation was configured to immobilize abundant capture probes. On the fabrication of typical sandwich hybridization, the designed biosensor was incubated with tetraoctylammonium bromide (TOAB). This TOAB incubation boosted the induced intrinsic redox activity of the tracer label and generated a discriminating current response. The optimized configuration exhibited a broad linear range functioning for 10 fM to 10 nM Mtb determination with a 3 fM LOD. In terms of selectivity, the designed sensor distinguished a mismatched DNA sequence and also differentiates Mtb from other pathogenic agents. The preliminary scrutiny of the clinical analysis revealed an excellent ability to recognize the PCR products of clinical samples. Thus, after the Hsp and ESAT-6 protein identification in earlier 2020 and 2019 studies, this is the third attempt toward identifying Mtb using its IS6110 fragment (Bai et al. 2019).
- In a noted 2018 study from the collaboration of Kazakhstan and the United Kingdom, Sypabekova and colleagues optimized the feasibility of a synthetic receptor in the form of an aptamer (a short stretch of oligonucleotides) for the detection of secreted protein MPT64, which is an acutely immunogenic polypeptide of *MTB*. Toward the sensing efficacy, the investigators screened the combinatorial effects of an aptamer linker and a co-adsorbent over an Au electrode for optimal binding efficiency and reduced nonspecific interactions for label-free

MPT64 detection using EIS. Two configurations of co-adsorbents and aptamer linkers were studied and high specificity and sensitivity to MPT64 were noticed for a surface prepared with a thiol PEGylated aptamer, HS-$(CH_2)_6$-$OP(O)_2O$-$(CH_2CH_2O)_6$-TTTTT-aptamer and 6-mercaptohexanol in 1:10 stoichiometry. The optimized configuration was evaluated for real-time significance via access to a spiked human serum sample with an LOD of 81 pM. This study, therefore, demonstrated the application suitability of MPT64 aptamer as a low-cost, accurate, and stable replacement of antibodies for the development of point-of-care TB biosensors, decreasing the detection time from several days or hours to 30 min (Sypabekova et al. 2018).

- Another study of 2018 is the study by Phan and colleagues, who designed a highly specific and accurate dot-blot immunoassay for the detection of culture filtrate protein (M_w: 10 kDa, CFP-10) through the formation of a Cu nanoshell on Au NPs surface. The optimized assay configuration worked through a Cu^{+2} reduction on the GBP-CFP10G2-Au NPs conjugate, possessing Au binding and antigen-binding affinities simultaneously, appearing as a red dot that could be noticed with the naked eye. The dot intensity was found proportional to CFP-10 TB antigen, with a 7.6 pg•ml^{-1} LOD. The analytical performance of optimized dot-blot configuration was studied as superior to that of a conventional Ag nanoshell. The probe efficacy toward real-life detection ability was also monitored via identification of CFP-10 antigen in urine samples, wherein minimum steps, high sensitivity, and specificity were observed (Tu Phan et al. 2018).
- Yet another 2018 attempt comprises an attempt from Malaysia, wherein yet again CFP-10 identification was made using a robust sandwich electrochemical immunosensor working through fabricated graphene/polyaniline (GP/PANI) nanocomposite over the screen-printed Au electrode. The configuration enabled an efficient detection of CFP10, with the Gp/PANI probe being characterized using FT-IR spectroscopy and FE-SEM. The morphology and chemical bonding pattern of GP/PANI nanocomposite were studied using FE-SEM and Raman spectroscopy revealed a successful coating of GP/PANI composite on the SPGE using a drop-cast technique. The coating of nanocomposite enhanced the SPGE surface area by nearly five times than the uncoated SPGE. The probe functioning was monitored and validated using CV and DPV analysis, wherein the CFP10 detection could be optimized. The optimized configuration exhibited a wide linear range (20–100) ng•ml^{-1} with an LOD of 15 ng•ml^{-1}, thereby conveying the sensitivity, rapidity, and disposable suitability of the designed configuration for TB detection within the real samples (Azmi et al. 2018). Studies like these serve as valuable databases for biosensing advances of graphene and related compounds. The results of this investigation must be compared with NPs' functionalized graphene to understand whether the composite framework could substitute an NP under certain conditions.
- Yet another significant attempt by Bakhori and colleagues reported a plasmonic ELISA method to study the antigen–antibody interaction using an ultrasensitive and affordable naked eye TB detection and diagnosis. The detection mechanism made use of the biocatalytic cycle of intracellular enzymes, which induces the

formation and successive growth of Au NPs. The detection of TB analytes in the sample solution was inferred by the formation of differently colored solutions by the plasmonic NPs in the presence of enzyme labels. The detection of disease was made using the standardized ELISA working that used catalase-labeled Abs, the enzymes consumed H_2O_2 and further generate Au NPs on the inclusion of $AuCl_3$. The blue or red color of the Au NPs-containing solution as a confirmatory indication for naked eye detection of TB analytes was determined based on the leftover H_2O_2 amount in the solution. This feature distinguished the optimized assay from conventional ELISA, which only develops tonal colors and requires a high analyte concentration to achieve distinguishing naked eye detection. The investigators also incorporated the MTB-specific ESAT-6 protein fragment in the optimized configuration for the detection of TB using plasmonic ELISA. The technique enabled the CFP-10 detection limit to as low as 0.01 $\mu g \cdot ml^{-1}$ on being visualized by the naked eye. The designed sensor was also studied on a confirmatory scale with the sputum samples from TB-positive patients, thereby providing enough evidence for a manifested suitability for early TB diagnosis (Bakhori et al. 2018).

9 Conclusions

Biomaterials exhibit immense potential for being used as sensing the interactions of the biological platforms. The structure-activity-specific functioning of these entities makes them competent for diversified activities. With advancements in characterization techniques and the emergence of shape and size-dependent functioning of NMs, the potential of biological sensing has evolved over a new horizon. The natural flexibility and self-assembly-driven sensing of biomaterials are widely instrumental in their roles as implicit detection moieties. Tailoring of NMs with biocompatible probes has been a major achievement of the emerging nanoscale phenomenon, wherein a precise detection of the slightest deviations in the properties has become possible. Many studies are in the final phase of being put on the commercialization scale, but there is no second path to meeting the set ethical guidelines, for which the procedures regarding reproducible outcomes have to be met. Such imbibitions manifest an urgent need of bridging the material sciences, electronics, and biological sciences so that smart biofunctional materials with steadfast and accurate responses could be developed. About mammalian cells, the whole tissues or specific regions are equally capable of being used as sensors, but appropriate measures must be considered to prevent any possible cross-reactions.

10 Cross-References

► Nanomaterials: Compatibility Towards Biological Interactions

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Nanomaterials: Compatibility Towards Biological Interactions

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Contents

Abstract

With the expansion of nanotechnology, health care sector has seen a boom in the field of diagnostics and therapeutics. Despite heaps of research, still the world of nanomaterials is illusive to us, especially its interaction with any biological system. This chapter is dedicated towards a basic understanding of the

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_19-2

classifications and characteristics of nanomaterials used in biomedical applications, the nano-cellular system interaction mechanism, and the factors governing their interaction. Another vital topic discussed in this chapter is the compatibility of these nanomaterials in any in vivo biological application in connection with biodegradability, cytotoxicity, accumulation in tissues and organs, and possibility of excretion from the body.

Keywords

Biocompatibility · Cytotoxicity · Nanomaterial interaction · Organic nanomaterial · Inorganic nanomaterial

1 Introduction

Nanomaterials are miraculous materials with unique properties that are distinctly different and enhanced to their own bulk counterparts. As of date, nanomaterial research in the fields of nanomedicine, nano-biology, and nano-toxicology as well as nano-diagnostics is growing rapidly and bringing in tremendous developments. As we talk about the role of nanomaterials in these biomedical applications, the interaction of nanomaterials with biological systems and their compatibility is extremely crucial to understanding the successful implementation of these nanomaterials. A clear insight on this interaction will facilitate to recognize the risk and the impact of nanomaterials on health and hence our life. There is a clear need to be concerned about the toxicity of nanoparticles; the potential long-term detriment nanomaterials could cause; because these are mostly newly explored, and many are still in the early stages of research with scary and positive evidence backed up by successful clinical trials. As nanomaterials interact with biological systems, there may be unpredicted biological effects that may be either adverse or favorable. There is a big question mark as to whether we should be concerned about the adverse effects or the potential compatibility of nanoparticles, or whether we should investigate and learn more to make the notion practically feasible.

The latest technologies have enabled the use of different characteristics properties at the nanoscale by the creation of nanomaterials in a controlled and refined manner. By controlling the size and shape of the nanostructures, nanotechnologies can also control the usage, design, arrangement, and their portrayal in biomedical fields. The use of nanomaterials has brought a revolution in the field of biomedicine by upgrading the prevention, detection, and treatment of diseases on cellular levels. The extent of functionalizing nanomaterials to alter their properties has made them an excellent resource for use in diagnosis and therapy of biological systems. Use of nanomaterials has given a significant breakthrough to solve critical problems relating to human health and to overcome age-related health issues (Azzawi et al. 2016). This is possible due to the compelling use of nanomaterials at cellular and subcellular levels. Various aspects of nanomaterials have been discussed in many research papers for a wide variety of biomedical application. This includes the study of drug expression in a particular population, use of nano-bots in medicine, their role in treating cancer, their use as

bioimaging agents, skin-contacting sensors, and nano-devices to detect activities at cellular and subcellular levels and many more (Azzawi et al. 2016).

However, there are several gaps that still need attention to analyze the numerous constraints pertaining to the knowledge of nanomaterials, their structure, synthesis method, and characterization. Another major challenge is the mass production of these nano-moieties that can reduce the cost of these nano-based medical applications. There is also a need for quicker and more accurate methods to control the desired properties of nanomaterials, which can be a boon for their use in biomedical fields.

Nanomaterials can be ingested, inhaled, or they can penetrate the skin readily. Hence, there is a growing interest in evaluating their potential toxicity in the body. As of now, study of nanomaterials causing toxic effects to the human health is limited. However, several efforts have been made regarding the same, but they have not been very much successful. Such efforts have led to the establishment of another subsidiary branch, known as "nano-toxicology." Several published research articles are dedicated to keep a check on the toxic effects of nanomaterials.

In this chapter, we have addressed the current gap in knowledge as well as the developments made in this interdisciplinary field of research. Aimed at this goal, an extensive classification of various types of nanomaterials is presented, majorly classified into inorganic – metal and metal oxide nanoparticles; organic nanomaterials and natural nanomaterials such as lipids, micelles, dendrimers, etc. Further, the chapter encompasses understanding of different types of possible interaction of nanomaterials with biological systems and various factors affecting this interaction. Finally, an insight into the compatibility in terms of biodegradability, cytotoxicity, and stability is portrayed. The summary includes a future assessment of the topic of discussion and its possible fate in near future.

2 Classification and Characteristics of Nanomaterials

Nanomaterials undergo several significant changes in a tangible environment. It is therefore required to study the state of the nanomaterials and their characteristic features prior to the interaction probe. Many nanomaterial-related studies focus on issues that have an impact on the characteristics of the nanomaterial, eventually on the cellular attributes and bio-dispersal, which in turn depend upon the shape and size of the particle. Adding to it, the size and shape of the nanomaterial are analytical for regulating cellular spiking, intracellular location, and bio-disposal. As shown in Fig. 1, nanomaterials can be broadly distinguished under the categories of inorganic and organic.

2.1 Inorganic Nanomaterials

A major classification of inorganic nanomaterials includes metallic nanoparticles, metal oxide nanoparticles, and semiconductor nanocrystals better known as semiconductor quantum dots. These nanomaterials with different shapes and dimensions have found loads of applications in almost every industrial sector including the

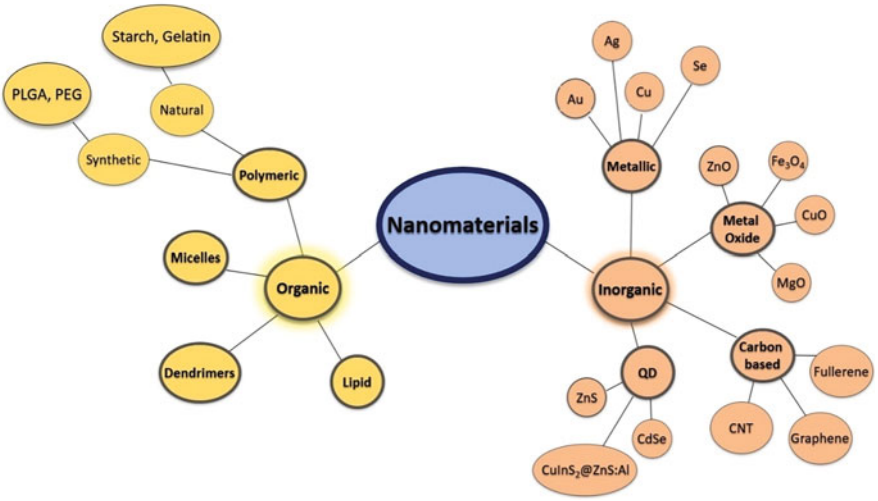

Fig. 1 Classification of nanomaterials

health care and medical sector. Although these nanomaterials are still in their R&D stage of development, they hold a big promise of commercialization in various biomedical applications very soon.

2.1.1 Gold

The use of gold (Au) in biomedical applications is a part of ancient medicinal knowledge in several parts of the world. Typically, biomedical use of Au nanomaterials is a broad and popular field of research. Au nanomaterials are known for their unique properties like chemical inertness and high stability, which allow excellent biocompatibility in both in vivo and in vitro applications. Au nanomaterials of specific dimension of less than 50 nm also exhibit excellent optical properties like surface plasmon resonance with chemiluminescence and fluorescence property. These nanoparticles are easy to functionalize to enhance the interaction with biomolecules for a particular application. This is possible due to the charge present on its surface. Also, Au nanomaterials are less invasive and less toxic to human beings.

Applications

Au nanomaterials have been explored in a wide range of applications. For instance, in targeted drug-delivery systems, anticancer therapies, contrast agents in imaging, molecular imaging, antimicrobial treatments, biosensors for disease diagnostics including cancer and several chronic diseases, and intercellular studies, hyperthermia-based therapies to treat malignant tumors, biocatalysts (Giljohann et al. 2010), etc.

2.1.2 Silver

Silver (Ag) is the most widely used nanomaterial worldwide. These materials can be made into several shapes, but spherical particles are the most commonly used configuration. The large surface-to-volume ratio of Ag nanomaterials enhances binding with many ligands. Ag nanomaterials have very high electrical conductivity, high stability, and low frit temperature. The large area of plasmonic field and tunable structures paves the way for various applications.

Applications

Ag nanomaterials have found their applications in various biomedical fields, primarily due to their ease of handling and holding of biomolecules (Gnanadesigan et al. 2012). These are used in biosensors and are considered one of the best catalysts. Nano-silver has a high efficacy of delivering genes and drugs to target specific sites and is widely use in theragnostic applications. Ag nanomaterials are known to be some of the best antibacterial, antiviral, and antifungal materials. Different nano-silver moieties have been used in wound healing and dressing applications. These materials are used in anticancer drugs and treatment. Ag nanomaterials are also used as implants for several body parts, for example, dental and bone implants. However, toxicity, potential efficiency, and costs remain a concern for their use in the human body.

2.1.3 Copper

Copper (Cu) nanomaterials have gained interest due to their high melting point, immense conductivity, low electrochemical relocation behavior, and low cost. Cu nanomaterials are much more reactive as compared to their bulk counterparts. Cu nanomaterials are highly stable and are seen to possess antimicrobial properties.

Applications

Cu nanomaterials have been explored in several biomedical applications. Typically, Cu nanomaterials have demonstrated significant antibacterial and antifungal effects. Cu nanomaterials have also been utilized in molecular imaging and cancer therapy. Photothermal ablation of tumor cells has been performed with the help of Cu nanomaterials (Badawy et al. 2015). However, the cytotoxicity of Cu nanomaterials is a concern.

2.1.4 Selenium

Selenium (Se) nanomaterials have gained attention and are successfully used in the fields of biomedicine and nutritional additives. Se nanomaterials play a key role in the formation of antioxidants like deiodinase. Se nanomaterials show scavenging effects due to their free radicals that can work efficiently in both in vivo and in vitro conditions. Se nanomaterials possess antimicrobial effects against some microbial species. Se nano-moieties have also demonstrated some anticarcinogenic action. Se-based nanomaterials show much lower toxic effects as compared to their bulk state.

Applications

Se nanomaterials have been used for intercellular assays. These nanoparticles have also been used in targeted drug delivery systems as drugs and as enzyme-carrying vehicles. Its role in biosensors and antimicrobial activities is also significant. Se nano-moieties are used in anticancer treatment as they can induce tumor cell apoptosis. Se nanomaterials have also been used in antidiabetic therapies with their ROS-scavenging abilities. Se nanomaterials also aid in food feed for mainly farm animals (Iranifam et al. 2013). They are used in food supplements to enhance growth performance and to improve the body's immune function via antioxidant effect. These particles have also shown their anti-inflammatory reactions to improve the efficiency of vaccines.

2.1.5 Zinc Oxide

Zinc oxide (ZnO) is present naturally inside the earth's crust as zincite. ZnO nanomaterials have demonstrated versatile applications, including excellent use as antifungal, antibiotic, and antimicrobial materials. ZnO nanomaterials, being semi-conducting, can communicate with electrons at a very high rate, leading to great electrochemical properties. ZnO nanoparticles have been validated with a high surface-to-volume ratio, nontoxicity, and chemical stability.

Applications

ZnO nanomaterials have been extensively used for their antimicrobial properties by incorporating them into bandages, coatings, agrochemicals, alloys, and textiles. ZnO nanomaterials are used as contrast agents in MRI (magnetic resonance imaging), as catalysts, and in medical biosensors due to their magnetic properties (Venu Gopal and Kamila 2017). ZnO nanomaterials are also extensively studied for their use as drug/gene delivery vehicles in the treatment of diseases. ZnO-based nanomaterials are known to be used for highly target-specific tumor cell destruction. These materials are used in drug delivery, bioimaging, and in the detection of tumors and are used in biosensors and as biomarkers for detection. In addition, ZnO nano-moieties are widely used in cosmetic products due to their efficient UV blocking capabilities.

2.1.6 Iron Oxide

Iron oxide nanomaterials have found tremendous use in various fields due to their chemical stability, low toxic effects, biocompatible nature, and large surface area with small size. Iron oxide nanomaterials exhibit superparamagnetic behavior and ease of modification with an applied field that allows these materials to be recycled or reused. The ease of separation of iron oxide nanomaterials due to their excellent magnetic properties is also a boon which enables their use in various biomedical applications. They are popularly known as SPIONS – superparamagnetic iron oxide nanoparticles.

Applications

Iron oxide nanomaterials are commonly used as contrast agents in MRI – magnetic resonance imaging. Iron oxide nanomaterials have been used in drug and gene delivery with an externally applied magnetic field. Iron oxide-based nanomaterials have been tested as antimicrobial agents. These materials have also been explored in cancer diagnosis, treatment, and therapy using external magnetic fields (Espinosa et al. 2016). Iron oxide nanomaterials have also been explored in tissue engineering and intercellular analysis. SPIONS are excellent materials when it comes to magnetic bio-separation.

2.1.7 Copper Oxide

Copper oxide (CuO) nanomaterials have gained interest especially due to their strong antimicrobial and biocide effects. CuO nanomaterials also have a unique blend of electrical, optical, and magnetic properties. These particles are highly stable with long shelf life and are cost efficient.

Applications

These materials are extensively used in wound healing applications and as microbial warfare agents. CuO nano-moieties are widely used as antimicrobial materials – antiviral, antifungal, and antibacterial. CuO nanomaterials are often used as catalysts and in cosmetic products. These materials are also used in different types of sensors – glucose sensors, immunosensors for detection of cancer, dopamine sensing, etc. Besides, the antioxidant and anti-inflammatory activities of the CuO nanomaterials are often utilized in various biomedical applications (Oliveri 2020).

2.1.8 Magnesium Oxide

Magnesium oxide (MgO) nanomaterials possess high solidity, high melting point, and high sterility. These are nontoxic and odorless materials. The high specific surface area and crystal structure are added advantages of these materials. The high surface reactivity, thermal stability, and chemical stability of MgO make it an encouraging material for various applications.

Applications

MgO nanomaterials have been investigated for bioimaging, coating of implants, wound healing and skin injuries, tissue regeneration, anticancer strategies (Sharma et al. 2017), and in drug and gene delivery applications. These materials have also been studied in nano-cryosurgery, as antimicrobial agents, and have also been applied as food additives and in food supplements.

2.1.9 Quantum Dots

Quantum dots (QDs) have acquired the captivating attention of researchers due to their extraordinary potential in biomedical and pharmaceutical sectors. QDs are semiconductor nanomaterials and show strong fluorescence under light sources like laser.

QDs can be fused via direct binding with biomolecules like nucleotides, proteins, and imaging agents, which are used for biosensing, bio-labelling, targeting, and imaging. Core shell quantum dots are used for a variety of applications in biomedical fields. The shell provides better bio-functionalization, dispersibility, and shows enhanced optical properties. $CuInS_2$@ZnS: Al, ZnS, and CdSe are some of the widely used QDs.

QDs have gained immense interest in bioimaging and bio-labelling applications because of significant benefits over the conventional organic fluorophores as listed below:

- QDs have high signal-to-noise ratio (S/N) compared to conventional organic dyes.
- QDs possess long fluorescence and notably high photoresistance.
- QDs are much better resistant to photobleaching, thus leading to an enduring photostability.
- These materials are roughly 10–15 times brighter than organic dye base fluorophore.
- QDs give sharp and narrow emission peak and broad excitation spectra.
- QDs can be easily sculptured into any shape, and a variety of biomolecules can be coated over these particles.
- By varying different aspects of QDs, like size of the core and its composition, shell composition, and exterior finish, their optical properties can be altered.

Applications

QDs find immense research interest in the field of life science. QDs are used in major imaging techniques like MRI (magnetic resonance imaging), nuclear and optical imaging. Different types of QDs can be used in various biomedical fields which differ in terms complexity, resolution, acquisition time, sensitivity, and cost. Direct imaging property of QDs can be used to overcome some disadvantages that conventional diagnostics methods possess. For instance, using QDs in cancer treatment for delivering the drug to the specific site and tracing its path with help of the imaging property of QDs. Functionalized QDs can be used in live cell operations as suitable probes. QDs are used in cells for biomolecular tracking, staining of tissues, tumor imaging, and vascular imaging (Schnee et al. 2012). QDs also have certain challenges that these materials are quite cytotoxic and the time of reaction is slow.

2.1.10 Fullerenes

Fullerenes are sp^2-hybridized carbon cages formed with double bonds and single bonds. Fullerenes are the most symmetric members of the carbon family and have chemical and structural stability. These materials are an important part of medicinal research due to their promising biocompatible properties. The structure, electronic configuration, size, and hydrophobicity of fullerenes have made them extremely appealing in the biomedical field. Its caged structure gives much scope for modification and functionalization, which makes it an efficient candidate for various

applications. Despite the low solubility of fullerenes in the physiological environment, they have attracted significant researchers.

Applications

Fullerenes can be used in radical scavenging and also as antioxidants. These materials can give a high quantum yield of singlet oxygen when exposed to light. With the use of this property, fullerenes can cleave DNA by directly transferring electrons from their excited state. These materials have been studied as carrier vehicles for drug and gene delivery. Fullerenes can inhibit the approach of substrates to the sites of enzymes. These can be used as excellent biomarker materials. Talking about the toxicity of fullerenes, data shows that pristine fullerenes are nontoxic. At the same time, many derivatives of fullerenes show toxic effects, although the degree of toxicity is moderate.

2.1.11 Graphene and its Derivatives

Graphene is a single layer of carbon with densely packed sp^2-hybridized atoms, forming a honeycomb-like lattice structure. It is often referred to as "wonder material." Graphene and its derivatives have gained significant research interest due to their excellent physiochemical properties. These materials have high mechanical strength, stretchability, flexibility, biocompatibility, and high impermeability. Graphene and its derivatives are chemically inert. The exceptionally thin structure and high surface area paves its way to be applied in several applications, where its bulk counterparts cannot be used. Graphene and its derivatives can be processed in aqueous conditions and are amphiphilic in nature. It presents ease of functionalization and can suppress fluorescence.

Applications

Graphene and its derivatives have been used as biosensing and imaging materials due to their selectivity, solubility, and outstanding biocompatibility. Gene and drug delivery with graphene and graphene-based materials has been successfully demonstrated due to their large surface area, high loading capacity, ease of membrane penetration, and high purity (Chung et al. 2013). These materials have found intriguing applications in cell growth, cell culture, and tissue engineering. These materials can also be used for bio-functionalization of proteins and in teeth and bone implantations. It is to be noted that graphene and its derivatives are toxic and can contain impurities due to methods of synthesis. Mass production of graphene-based products is a tedious work and is cost-consuming. Moreover, it is difficult to control the thickness and size of graphene materials.

2.1.12 Carbon Nanotubes

Carbon nanotubes (CNT) are hollow and ordered carbon-based nanostructures arranged in the form of cylinders. These carbon atoms have sp^2 hybridization. It is classified into two basic types based on the structural formation: single-walled and multi-walled CNT. Single-walled CNTs are a single layer of carbon atoms rolled into a cylindrical shape, whereas multi-walled CNTs contain many sheets of carbon

rolled into a cylindrical shape in a concentric manner. CNTs are highly elastic and very flexible. CNTs have magnificent mechanical strength and high thermal and electrical conductivity. The high aspect ratio and high surface area of CNTs are utilized in various applications. CNTs can be functionalized easily by covalent and non-covalent methods. Modification of CNTs can be done on their surface and tip ends. By modifying the structure, various functional groups can be added to the CNTs, which makes them useful in almost every field.

Applications

CNTs have been employed in targeted gene and drug delivery systems as they can penetrate easily through the cells and keep the cargo intact. CNTs have often found their use in electrochemical biosensors for detecting various biomolecules and in tissue engineering. These materials have also been broadly investigated for diagnostic purposes. CNTs have immense potential in bio-medicinal fields. Being small and light in weight, CNTs can go well in the air and can be inhaled, causing toxic effects. CNTs can also cause dermal toxicity and lead to skin diseases. However, their toxicity depends upon the shape, structure, and the functional groups attached.

2.2 Organic Nanomaterials

Although inorganic nanomaterials are being extensively explored in biomedical applications, a major concern of cytotoxicity limits their uses in in vivo applications. With substantial interest in biocompatible and nontoxic nanomaterials, many organic nanomaterials, particularly polymeric nanomaterials, are being broadly explored.

2.2.1 Micelles

Micelles are aggregates of amphiphilic block copolymers or lipid molecules in a spherical shape. These structures are formed by the self-assembling of polymeric molecules upon the surface saturation of the solution at a particular polymer concentration. This concentration, above which polymeric entities begin assembling as micelles, is known as the critical micelle concentration (CMC). The hydrophobic chain of the amphiphilic polymer starts to aggregate, decreasing its water contact, forming a central core and hydrophilic part pointing outwards to the aqueous medium, forming the corona/shell of the micellar structure. Various copolymers in the form of di-block and tri-block, including polyethylene glycol (PEG), poly (N-isopropyl acrylamide) p(NIPAAm), Poly(ϵ-caprolactone) (PCL), poly(N-vinyl pyrrolidone) (PVP), and poly(lactic-co-glycolic acid) (PLGA), lead to the formation of micellar structures. The corona, being the outer part of the micelle, is responsible for determining its biocompatibility, circulation half-life, and stability in the environment. Subsequently, it also helps in determining the efficacy of micelle by interacting with the cells and other biological components of the blood for use in biomedical applications. On the other hand, the central core of the micelle is used for the loading of drugs. The core being hydrophobic in nature allows only the hydrophobic drugs to be loaded inside it. This helps in increasing the water solubility and

decreasing the toxicity of the hydrophobic drug. Interestingly, the shell/corona of micelles helps in keeping the micelles stable for a longer duration in plasma because of their hydrophilic nature and prevents their opsonization, resulting in their escape from the reticuloendothelial system (RES), subsequently avoiding the pre-degradation of drug and increasing its efficiency. Along with this, the nano-size of micelles allows easy penetration and accumulation into the tissues, which results in a high concentration of drugs at the target site by the enhanced permeation and retention effect (EPR), mainly observed around the tumor regions. Additionally, a higher drug loading capacity is seen in the case of micelles. Despite several benefits, use of micelles for drug delivery is obstructed by the fact that upon dilution, micelles tend to lose their shape, which leads to undesired release of the drug. To overcome this, amphiphilic copolymers having a low CMC value are used to prepare micelles or they are stabilized by post-polymerization after the self-assembly of monomers, which would help in maintaining a concentration equilibrium between the solution and the micelle.

Applications

Drug delivery – In today's era, it is estimated that more than 70% of all the entities that have been researched upon for use as drugs are hydrophobic in nature. Thus, it becomes necessary to develop methods to increase their water solubility for human use. Polymeric micelles are one such solution to overcome this problem by acting as carriers for hydrophobic drugs. Hydrophobic drugs are loaded inside the core of micelles, and their outer shell helps in increasing the water solubility and decreasing the toxicity of the drug. Many experimental studies have shown the increase in drug water solubility of 10–8400 times. For example, paclitaxel loaded in poly(D, L-lactide)-methoxy PEG micelle showed 5000 fold times increase in the water solubility of paclitaxel drug (Burt et al. 1999). With the increase in water solubility and the nano-size of micelles, the absorption of drug inside the cells becomes more effective. It is also possible to modify the outer shell of the micelles using different kinds of ligands such as antibodies, antigens, genes, aptamers, or nucleic acids to enhance their biological properties. With the help of ligand modification, micelles can cross the blood-brain barrier by helping in transcytosis (transport of molecules from one side of a cell to other) to cross epithelial cells or by using Pluronic copolymers which inhibit P-glycoprotein for penetration of drug molecules into bovine mammary epithelial cells. These ligands also make target-specific delivery possible. One of the active areas for the use of polymeric micelles as drug delivery carriers is in tumor treatment. Micelles increase the solubility of hydrophobic anticancer drugs and their permeation inside the tumor cells by enhancing permeation and retention effects. Genexol – a polymer micelle US FDA approved drug – is used for breast cancer treatment.

Diagnostics: Contrast agents play a crucial role in imaging techniques such as magnetic resonance imaging (MRI), computed tomography (CT), and nuclear imaging. Contrast agents in micellar forms have been shown to be more advantageous than conventionally used contrast agent molecules because of their high biocompatibility and ability to be tuned according to our area of use. MRI contrast agents use

pH-sensitive micelles to detect cancer. These exhibit great contrast enhancement in low pH biological compartments and simplify the detection of very small size tumors as well in vivo. Micelles can simultaneously carry anticancer drugs as well as imaging probes for the detection of cancer, showing theragnostic (diagnostics and therapeutics) properties. Similarly, a CT scan applies contrast agents such as iodine, which are able to absorb X-rays for imaging in high resolution. To overcome the side effects of such heavy elements, polymeric micelles can be used. For example, PLL-PEG polymer micelle containing iodine was developed (Movassaghian et al. 2015) that helped in improving the circulation of iodine and decreased the amount of iodine used in maintaining the same contrast.

2.2.2 Dendrimers

Dendrimers are architectural motifs with well-defined hyperbranched symmetrical structures of polymeric molecules. It contains a small polymeric core surrounded by branching units representing a "branching tree" shape. The three main parts of the dendrimer structure are the central core, repeated internal branching units, and the terminal functional groups. These are self-assembled structures formed by the combination of different types of interaction such as electrostatic, drug-polymer interaction or complexes with nucleic acids. Additionally, unlike micelles, their self-assembly is independent of the concentration, which makes them more structurally stable in in vivo conditions. The structure of dendrimers highly resembles with many proteins, which makes dendrimers a promising material for use in medicine. The third generation of PAMAM dendrimers, for example, resemble insulin in size and shape, which makes them potential synthesizers of protein scaffolds. The size and shape of dendrimers can be altered with successive generations (conjugation of further monomer molecules) during their formation. Alongside, dendrimers are also being used as delivery vehicles, contrast agents, and as biomimicking agents in the sensors. Drugs are loaded within the core and polymer branches can be functionalized with various antibodies, molecules, and genes resulting in multi-functional dendritic structures. In some cases, dendrimers act as "*prodrug"* by forming covalent linkages with biomolecules and perform their activity only at the time of interaction with a cell. Typically, dendrimers are much more preferred over liposomes and polymeric micelles because of their in vivo uniform dispersion and stability.

Applications

Drug and nucleic acid delivery – The high surface area of dendrimers offers the possibility of delivering both hydrophilic and hydrophobic drugs using them. The hydrophobic drugs are loaded in the core of the dendrimer, and hydrophilic drugs are attached to its branches. Dendrimers can easily deliver drugs through oral as well as nasal pathways due to their nano-dimension and high solubility. PAMAM dendrimers show potential in this type of drug delivery. Additionally, target specific delivery is carried out using dendrimers as their polymeric branches are highly acceptable to modifications using specific antibodies, ligands, and other biomolecules as well. These modifications also allow a slow and regulated delivery of drugs.

For example, it was seen that when cisplatin was encapsulated in PAMAM dendrimers, a long-term slow release of cisplatin was achieved (Gupta and Nayak 2015). Similarly, studies are going on to treat genetic disorders by transferring genes using dendrimers (Lyu et al. 2020). siRNA widely helps in many therapeutic applications, but its use has become limited because of its unstable nature and easy degradation.

Artificial proteins – Because of their close resemblance with some of the protein structures, like hemoglobin and insulin, dendrimers are referred to as "artificial proteins." The high molecular weight and the hyperbranched structure of dendrimers are two contributing factors for their biomimicking properties. Dendrimers with structures like proteins inhibiting angiogenesis are being developed for their use in cancer treatment. These dendrimers act as proteins and inhibit the growth and differentiation of tumor cells.

Bioimaging – Dendrimers are used in contrast agents for MRI and CT techniques to improve the performance of contrast agent elements. In MRI, signals are generated by the relaxation rate of water protons in vivo. To enhance these signals and obtain a better result, contrast agents are incorporated inside the human body. One of the mainly used contrast agents in MRI is gadolinium (Gd) chelates. However, toxicity and biocompatible concerns are related to their use in in vivo conditions, and thus Gd is incorporated into the dendrimers to improve its circulation time and higher signal. Gd chelates are attached to the dendritic branches for their easy interaction with the protons that result in the MRI images. Similarly, for optical imaging purposes, fluorescent dyes can be attached to the dendrimers which would produce light on interaction or reaction at the specific sites resulting in efficient diagnosis technique.

2.2.3 Lipid Nanoparticles

Liposomes are phospholipid bilayer structures that are being extensively exploited as the drug delivery and gene delivery carriers. A simple liposome typically comprises of a hollow core of diameter ~ 50 nm to 1 μm surrounded by phospholipids bilayers. The hollow core acts as an accumulation site for therapeutic molecules. The number of phospholipid bilayers further classifies liposomes into different configurations such as multi-lamellar, small unilamellar, and large unilamellar. As the name implies, multi-lamellar vesicles are made up of several lipid bilayers that are separated from each other via aqueous spaces, whereas unilamellar vesicles have a single bilayer with entrapped aqueous regions. This unique biphasic nature of liposomes allows delivery of both hydrophobic as well as hydrophilic drugs congruently. Drug delivery system using lipid nanoparticles conventionally adopt the methodology of enhanced permeability and retention (EPR) effect. The EPR effect was first introduced by *Morgan* et al., where he first reported in vivo liposome imaging of deep-sealed infections and solid tumors using Indium 111 radiolabels (Morgan et al. 1985). Further, the discrete structural configuration, flexibility, and size of liposomes allow them to easily pass through the cell membrane followed by releasing the carried content, and thus, they can be recognized as smart carriers for targeted drug delivery. It is seen that liposomes have a short circulation half-life; to

overcome this drawback, liposomes are conjugated with polyethylene glycol (PEG) polymer, which helps in increasing the overall stability of liposomes.

Applications

Cancer therapy – Most of all the available anticancer drugs till date are highly toxic and hydrophobic in nature. These drugs not only affect tumor cells but also the nearby healthy cells. Liposome-based delivery system has great potential in cancer therapies. The encapsulation of anticancer drugs in liposomes helps in increasing their solubility and reduces the risk of toxicity by preventing leakage of drugs at undesired locations. Several anticancer-based liposomal formulations have already been approved by the FDA for use in cancer therapies. Doxorubicin encapsulated in PEGylated liposomes was the first liposomal delivery system approved by the FDA in 1995. Many other ligand-modified liposomal formulations are also under clinical tests.

Microbial infections – Fungal infections, though rare, have a high mortality rate. Amphotericin B is a standard drug used to treat a wide spectrum of fungus. Regrettably, toxicity risks are involved with its use, for which its encapsulation in liposomes is carried out. A liposomal formulation of Amphotericin B, AmBisome, was prepared which showed a high decrease in toxicity level while maintaining the same effectiveness of the drug (Faustino and Pinheiro 2020). Researchers are investigating to prepare oral formulations of AmBisome, as the presently available is costly and requires medical administration during its use (Faustino and Pinheiro 2020). Another serious problem related to microbial infections is the increase in resistant bacteria against the antibacterial drugs. With the frequent use of antibiotics, bacteria have started developing resistance, which leads to a decrease in drug potency. Liposomal formulations are seen to be a promising solution to maintain the drug efficacy. Arikace, an FDA approved Amikacin liposome-encapsulated formulation, is used against *Pseudomonas aeruginosa* and other lung diseases.

Gene delivery – Small-sized liposomes containing cationic lipids are found to be effective delivery carriers for DNA and RNA due to their low surface charge and long circulation time. Cationic lipid-based liposomes can easily encapsulate nucleic acids with a negative charge at low pH. Lipofectin™ is an effective, commercially available liposome prepared using dioleyloxypropyl-trime-thylammonium bromide (DOTMA) for DNA transfer. The positive headgroup of DOTMA forms a complex with the negative phosphate group of DNA and helps in transfection in cells.

2.2.4 Starch

Starch is one of the most common polysaccharides, produced by plants for energy storage. Nowadays, starch is being used as a biodegradable, nontoxic polymer for varied applications in the form of starch nanoparticles. Typically, starch nanoparticles are built from amylose and amylopectin in crystalline and semicrystalline structure. The physiochemical properties of starch nanoparticles depend on the source from which starch is obtained such as potato, wheat, and maize, because starch in its native form possesses some limitations which are overcome by using modified starch nanoparticles. Some of these are oxidized starch, cadexomer iodine

starch, hydroxyethyl starch having improved solubility, stability, and properties to pass through biological barriers. Cadexomer iodine is physically entrapped in the starch matrix which is hydrophilic in nature. This is used for the slow release of iodine onto the wound with the help of its pores. On the other hand, oxidized starch is prepared under specific environmental conditions using an oxidizing agent. It forms a highly stable film which can be used as a binder in the paper industry. Additionally, hydroxyethyl starch is produced by a reaction between starch and ethylene oxide to be used as plasma expanders for the treatment of articular perfusion. Additionally, since starch is derived from plants, it is inherently biocompatible, which favors its use as nanofillers for scaffolds in tissue engineering along with drug delivery. Studies have also shown that the coating of starch on inorganic nanoparticles such as PbSe nanoparticles renders it nontoxic and biocompatible (Torres and Arce 2015).

Applications

Scaffolds – Various studies show that starch nanoparticles when used as nanofillers enhance the structural properties. These are used as bone cements to provide temporary supports. Scaffolds are prepared using starch-based polymers which support the growth of epithelial cells on their surface for new bone formation and are later degraded into nontoxic residues. A nanocomposite of multi-walled CNTs and starch nanoparticles was developed as tissue scaffolds for regenerating bones (Famá et al. 2011).

Drug delivery – Starch nanoparticles have been explored as nanocarriers for drugs, increasing their absorption and bioavailability in the human body. Curcumin when delivered using starch maleate nanoparticles showed 300 times increase in curcumin solubility as compared to free curcumin (Pang et al. 2014). Similarly, indomethacin loaded in starch nanoparticles showed an improved pharmacokinetic behavior (Simi and Emilia Abraham 2007). It is also seen that starch nanoparticles permit controlled drug release in both acidic and basic mediums.

2.2.5 Chitosan

Chitosan, a linear polysaccharide, is derived from the chitin of seashells. Chitosan nanoparticles are polymeric self-assembled nanoparticles that are highly biocompatible and biodegradable in nature. However, chitosan is water insoluble and poses significant hurdles regarding various applications, but its modification using functional groups helps in overcoming this limitation and widens the area of application. The amine groups of chitosan are responsible for its various physical and chemical properties. Literature shows that chitosan nanoparticles help in achieving antitumor activities by improving the body's immune functioning (Nanoparticle et al. 2019). Besides, chitosan is a cationic polymer, which helps it adhere to bacterial cell walls. This adherence prohibits further bacterial growth, showing great inherent antibacterial properties. According to studies, chitosan with a low molecular weight is more effective against gram-negative bacteria (Younes et al. 2014). Chitosan nanoparticles also show antioxidant properties by scavenging reactive oxygen species, preventing the damage of proteins and other lipid membranes. Enzymes present in

biological organisms can easily depolymerize chitosan, which prevents its accumulation inside the body, making them highly nontoxic in nature.

Applications

Antimicrobial agent – Chitosan nanoparticles, because of their cationic nature, show antibacterial activity against a wide range of bacteria. These can enter the cell wall of bacteria and inhibit DNA transcription and mRNA synthesis. Studies show that the type of bacteria affected by chitosan nanoparticles depends on its molecular weight, surrounding pH, and then its acetylation (Zeng et al. 2014). The low pH and molecular weight of chitosan favor antimicrobial activity against gram-negative bacteria because of more negative charge on cell wall leading to strong interaction between chitosan and bacteria. Chitosan nanoparticles also showed antifungal activity against *Fusarium oxysporum*, which increases by increasing the molecular weight and deacetylation of chitosan.

Wound healing – ChiGel, Tegasorb™, and ChitoFlex are some of the commercially available forms of chitosan for use in the wound healing process. Chitosan nanoparticles, being charged particles, attract the red platelets to speed up the coagulation at the site of the wound. These are also seen to show macrophage movement and enact cell expansion. Along with speeding up the healing process, chitosan nanoparticles prevent any bacterial infection around the wound. Chitosan-based nanofibers are used as cement-based chitosan for dressing material. Chitosan, in the form of scaffolds and sponges, is also used in wound healing applications.

Drug and gene delivery – Chitosan nanoparticles are able to carry drugs as well as nucleic acids for delivery. Chitosan is viscous in nature, which helps in the slow release of drugs, improving their therapeutic efficiency. Chitosan nanoparticles have proven to be good candidates for oral and nasal delivery of drugs, as in the microsphere form, these particles are easily absorbed by epithelial cells.

2.2.6 Gelatin

Gelatin is one of the important natural polymers used in various applications. The wide scope of using gelatin is possible because of its biocompatibility, biodegradability, readily accessible, and economical properties. Gelatin is formed by either acidic or basic hydrolysis of collagen in two forms: Type A and Type B gelatin nanoparticles. The isoelectric point of Type A gelatin is around seven, whereas for Type B gelatin it is around four, and among these, Type B gelatin nanoparticles exhibit better efficiency of drug delivery. The US FDA has approved gelatin as Generally Recognized as Safe material for its use in in vivo applications such as tissue engineering and drug delivery systems. Gelatin nanoparticles can effectively carry drugs and help in their enhanced delivery.

Applications

Drug and gene delivery – Gelatin nanoparticles are promising nanocarriers for both drugs as well as genes. These assist in reducing the toxic effects of drugs and also in increasing their biocompatibility. Drugs travel easily across the plasma without getting pre-degraded. By enhanced permeation and retention effects, gelatin

nanoparticles accumulate a high concentration of drug on the target sites. It was seen that the toxicity level of noscapine decreased considerably by loading it into gelatin nanoparticles (Madan et al. 2011).

Tissue engineering – Gelatin, being a denatured polymer, offers an advantage over other biopolymers in making scaffolds for tissue engineering purposes. Gelatin nanocomposites are formed with greater mechanical strength for bone scaffolds. A study showed that bone can easily penetrate inside the scaffold made using gelatin and hydroxyapatite composite because of its high elastic nature (Venugopal et al. 2008). Studies prove that composites with gelatin conjugation are one of the best materials for bone regeneration application (Peter et al. 2010).

2.2.7 Synthetic Polymeric Nanomaterials

Poly(Lactic-Co-Glycolic Acid)

Poly(lactic-co-glycolic acid) (PLGA) is one of the most successful synthetic polymers to be used in the biological applications approved by the US FDA. The main reason is its biodegradability. PLGA nanoparticles are hydrolyzed into lactic acid and glycolic acid which are then metabolized into carbon dioxide and water via Krebs cycle. Additionally, the surface of PLGA can be easily decorated using various ligands such as antibodies, genes, and other biomolecules. PLGA is a copolymer of polyglycolic acid (PGA) and polylactic acid (PLA) linked by ester linkages. The ratio of these monomers determines the molecular weight and degradation time of PLGA nanoparticles. Alongside, by varying the monomer ratio, PLGA can be made into any configuration for carrying a wide variety of biomolecules.

Applications

Typically, polymeric nanoparticles are used for drug delivery and therapeutic purposes, exhibiting stimuli-responsive activity and stability in the extreme pH and temperature conditions of the human body.

Drug delivery for cancer therapy – PLGA nanoparticles can encapsulate hydrophobic as well as hydrophilic drugs for their efficient delivery. In case of cancer treatment, PLGA nanoparticles are used to increase the solubility and efficiency of hydrophobic drugs such as doxorubicin. For instance, to treat breast cancer, salinomycin and paclitaxel were encapsulated in PLGA nanoparticles and further anti-CD133 antibodies were grafted on their surface for targeting breast cancer cells and on-site release of the drugs (Swaminathan et al. 2013). PLGA nanoparticles have also been explored for pH and heat-responsive drug release.

Imaging and diagnosis of cancer – Imaging plays a vital role in keeping a check on the recurrence of tumors and also monitoring any therapeutic response. Alongside, it is also important to diagnose cancer at an early stage for efficient treatment. For this purpose, PLGA nanoparticles containing superparamagnetic iron oxide were prepared to be used as contrast agents in MRI (Wang et al. 2008). In another study, radiotracer technetium-99 m was encapsulated in PLGA nanoparticles for the detection of sentinel lymph nodes (Acharya and Sahoo 2011).

Polyethylene Glycol

Polyethylene glycol (PEG), also called as stealth polymer, is one of the widely used polymers in drug delivery applications. It is highly biocompatible, water soluble, biodegradable, nontoxic, and nonimmunogenic in nature. For use in various biomedical applications, PEG with different molecular weights and shapes are considered, depending upon the field of application. Typically, three shapes of PEG are used, i.e., linear, branched, and Y-shaped polyethylene glycol. Linear PEG contains one functional group which undergoes conjugation with protein on one side of the chain and a methoxy group on the other side. Two or more linear PEG together makes branched PEG structure which shows better immunological and pharmacokinetic properties. On the other hand, Y-shaped PEG shows more stability against pH, temperature, and other physiological environment (Mohapatra et al. 2019). The chain of PEG molecule does not contain any bulky group which makes it structure flexible. It also allows binding of water molecules leading to more hydrodynamic volume. The properties of PEG nanoparticles not only make it a versatile nanocarrier but also a good coating material for other nanoparticles termed as PEGylation. As a coating, PEG makes the nanoparticles hydrophilic due to the presence of repeating units of ethylene glycol. Additionally, PEG enhances the stability by reducing the charge interaction between protein and nanoparticles. It also acts as a barrier against immune system increasing the circulation time of nanocarriers.

Applications

PEG nanoparticles are majorly used as drug delivery carriers for both hydrophobic as well as hydrophilic drugs. Drug molecules are conjugated to PEG nanoparticles using linkers which on reaching the target site initiate their release. In one of the studies, camptothecin (CPT) was conjugated with PEG nanoparticles to form a CPT-PEG prodrug complex (Yu et al. 2005). CPT is an enzyme inhibitor which on binding with topoisomerase and DNA inhibits relegation of DNA and tumor cells are destroyed. But its use is limited because of low water solubility and toxicity to normal cells. To overcome this issue, CPT-PEG complex was formed in which PEG nanoparticles helped in making CPT water soluble and less toxic. Studies involving loading of doxorubicin (DOX) drug in PEG nanoparticles have shown to enhance its antitumor activity and prevent pre-degradation during its use (Veronese et al. 2005). Another area of application for polyethylene glycol is to coat metallic nanoparticles such as gold and silica with a layer of PEG. The coating of PEG improves the biocompatibility of such nanoparticles and protects them against various biological barriers during in vivo applications.

Poly-Ɛ-Caprolactone

Poly-ϵ-caprolactone (PCL), a biodegradable polymer, is exploited for its use in biological fields such as tissue engineering, wound dressing, and drug delivery. In addition to drug delivery, PCL is also used to deliver other proteins, vaccines, and biomolecules for the treatment of various diseases. Despite these advantages, PCL is not used as much as compared to other polymeric nanoparticles such as PLGA and PGA because of its slow degradation rate. PCL is highly stable as compared to other

biodegradable polymers because of a smaller number of free ester bonds which lowers its degradation rate. Although this property of slow degradation helps PCL nanoparticles to be used effectively in the making of implants and bone tissue that need the material with very slow or no degradation (Woodruff and Hutmacher 2010). Studies on the toxicity of PCL nanoparticles showed that they are non-mutagenic and safe for use.

Applications

Scaffolds in tissue engineering – PCL is the most favored nanomaterial for the use as scaffolds in tissue engineering because of its slow degradation rate which matches with time required for bone ingrowth and repair. Also, the pore size and interconnection are important during the development of scaffolds as they should not permit cell movement across them but also at the same time should allow the blood vessel growth inside them, and this can be easily achieved using PCL which allows easy tenability in this structure during preparation. Along with PCL, ceramics are used as fillers in them to enhance the mechanical stiffness of scaffold. Additionally, PCL nanoparticles are also being explored for the wound repair in soft tissues of the human body. It is seen that nanofibers of PCL help in improving the mechanical strength and cell interaction when used as fillers in natural polymers like chitosan.

Drug delivery – PCL nanoparticles are preferred for a long-term slow release of drug in the system. PCL, due to its hydrophobic nature, easily encapsulates hydrophobic drugs, whereas hydrophilic drugs are adsorbed on their surface. PCL nanoparticles help these drugs to pass through blood-brain barriers and the immune system, further allowing easy interaction with cell membranes to deliver drugs inside the cells. For example, carboplatin drug was loaded inside PCL nanoparticles using solvent-emulsion evaporation method for the brain delivery (Karanam et al. 2015). Studies showed that PCL nanoparticles loaded with curcumin drug exhibited slow- and long-term release of the curcumin drug (Espinoza et al. 2020).

Polylactic Acid

Polylactic acid (PLA) is an aliphatic, biodegradable polymer made up of lactic acid monomers. PLA can be easily derived from carbon dioxide, rice, wheat, which makes it very environmentally friendly. Along with this, PLA nanoparticles are very biocompatible, recyclable, and easy to produce, which makes it an excellent candidate to be used for biological applications. According to the area of use, it is easy to tune the mechanical properties of PLA. It is seen that sometimes the hydrophobic nature of PLA is responsible for the inflammatory response of the tissue.

Applications

Tissue engineering – PLA is used to form porous scaffolds due to their good mechanical strength and biocompatibility. These scaffolds are used to initiate the regrowth of tissues by acting as supports for them. A composite of PLA along with hydroxyapatite has been used to initiate osteogenesis and be used in dental

applications (Kim et al. 2020). PLA scaffolds are also used for the growth of epithelial cells. PLA along with PGA showed good results for this purpose (Kim et al. 2020).

Drug delivery – PLA nanoparticles are used as nanocarriers for delivery of drugs used in the treatment of various diseases. A major benefit of using PLA in drug delivery systems is that it easily dissolves in extracellular matrix and allows the delivery of drugs in a slow and controlled manner for prolonged effect. Studies showed a greater amount of drug accumulation at the target site when docetaxel was encapsulated in PLA nanoparticles as compared to when it was used as a free drug (Hrkach et al. 2012).

3 Interaction of Nanomaterials with Biological Systems

3.1 Governing Factors

There are various factors that can modify the conformation of nanomaterials and can be a potential cause for deteriorating their biological properties. The biophysical properties of nanomaterials display their recognition and effect on artificial moieties contained within, among all comprising morphology, chemical framework, solubility, shape and structure, size, and their aggregation status. These features also play a key role in studying specific mechanisms like cell biocompatibility. Some of the vital factors that strongly affect the interaction of nanomaterials on any biomolecules are: (a) interaction of the nanomaterial with its own surroundings, that is with other neighboring nanomaterials; and (b) physicochemical property and design architecture of the nanomaterial, for instance, size, shape, surface modification, protein corona effect, etc., as shown in Fig. 2.

3.1.1 Size

The size of nanomaterials plays a pivotal role in biological interactions with external systems and other biological mechanisms in the nano-regime, like cellular uptake and the processing effectiveness of the particle. The smaller the size of the nanoparticle, the faster will be its ionic release rate and interactivity with cell structures. The toxicity of nanomaterials is said to be directly dependent upon their size. A decrease in the particle size leads to an increase in the surface area to volume ratio, implying that the area of contact for the nanomaterial increases. Thus, its probability of invading into a cellular system gets higher and thereby increases the toxicity level.

It is clear that the size of the nanomaterials is extremely crucial in any in vivo administration or medicinal behavior. Nanomaterials greater than 1 μm cannot penetrate the cell membrane easily, but they can be linked to proteins absorbed in the cell. It has been reported that nanomaterials more than around 6 nm in dimension have a high chance of getting accumulated in different organs such as the kidneys where they cannot be further filtered. There are other studies reporting hepatotoxicity caused by CdSe quantum dots (Sanford et al. 2004). Other studies have investigated bioaccumulation and bio-dispersibility of different sizes of gold nanoparticles in the

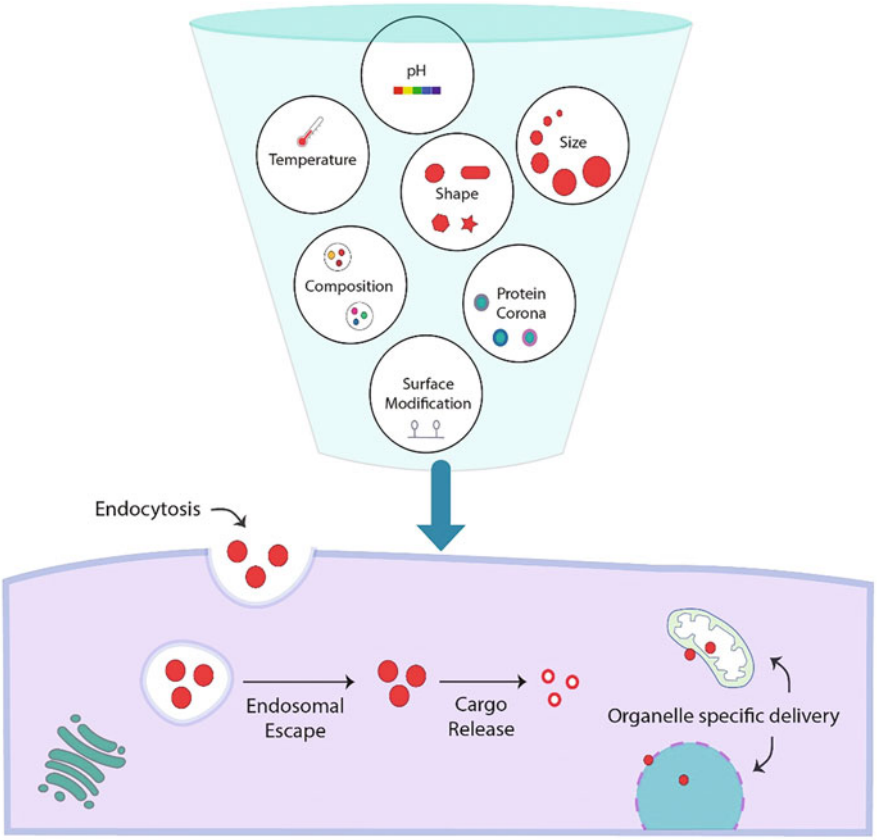

Fig. 2 Factors governing interaction of nanomaterials with biological systems

bloodstream and indicated that smaller gold nanoparticles stayed for longer durations in the blood and were also accumulated to a greater extent in the organs (Sonavane et al. 2008).

3.1.2 Shape

The toxicity of nanomaterials is also determined by their shape. Nanomaterials can be synthesized in different morphologies such as spheres, clusters, fibers, planes, tubes, and many other irregular shapes (Lee et al. 2019). Processes like phagocytosis, absorption, bio-dispersal, and elimination are highly influenced by the shape of the nanomaterial. For instance, the spherical nanomaterials have been found to be comparatively less toxic as compared to other shapes. Moreover, spherical particles with an even size distribution are absorbed faster as compared to rod-shaped materials.

3.1.3 Surface Modification

The nature of a nanomaterial's surface is strongly responsible for its solubility and cell-to-nanomaterial interaction. A surface coating or modification can immensely affect the pharmacology, dispersal, aggregation, and toxicity of any nanomaterial (Favi et al. 2015).

Phenomenon like *colloidal conduct, protein-plasma binding*, *absorption*, and *crossing of blood-to-brain barrier* are known to be influenced by the surface chemistry of a nanomaterial. For example, acetylation of starch nanoparticles leads to cross-linking, which in turn decreases the solubility and swelling property of starch. An increase in the charge at the surface of a nanomaterial leads to its increased cytotoxicity. This indicates that greater cell interactions occur due to high charge accumulation and, subsequently, more endocytic metabolism. It is proven that higher toxicity is caused by uptake of positively charged nanomaterials as compared to negatively charged ones. Other studies have verified this fact where positively charged nanomaterials were observed to aggregate more in tumor cells as compared to the negatively charged nanomaterials (Hoshyar et al. 2016). Another study has shown surface charge-mediated negative effect on the adsorption rate of chitosan nanoparticles on different biological uses during drug delivery and other uses. Precise surface modification of nanomaterials may also lead to increased stability, a decrease in toxicity, and controlled and modulated cellular incorporation. Organic moieties like amine, hydroxyl groups, PEG are often used in surface modification.

3.1.4 Protein Corona

A corona is formed around the nanomaterials when they encounter different biomolecules in the bloodstream. The corona formed by proteins mainly consists of proteins with a varied nature of interactions: *clusterin, fibrinogen, immunoglobulin, albumin,* and *apolipoproteins*. Adsorption affinity of the nanomaterials on the protein surface plays a vital role in the corona formation.

The physicochemical properties and the biological specifications of nanomaterials change once the protein corona is formed around them. The characterization of the protein–nanomaterial interaction is challenging. But an in-depth understanding could bring awareness about the negative impacts that corona formation can have on the activity of nanomaterials.

According to the Vroman effect (Hirsh et al. 2013), corona formation is initially restricted to surface proteins with a higher congregation and lower binding affinity and then with high affinity proteins in a lower congregation. Protein corona is of two types: hard and soft. Hard corona has more binding affinity and a longer time of exchange. It is also closer to the nanomaterial surface. The soft corona layer has lower binding affinity and a fast time of exchange. Various techniques used in the characterization of protein-corona interactions include SDS-PAGE, UV-vis spectroscopy, centrifugation, special chromatography techniques, and calorimetry.

3.1.5 pH Response

pH plays an important role during the synthesis of various nanomaterials as well as affects some of their physiochemical properties. Gold nanomaterials are better synthesized in a low pH medium. pH value also affects gold nanoparticles' bonding with various organic functional groups and biomolecules, changing their biological activities. Similarly, iron nanoparticles are observed to be suitable for bioimaging purposes in an environment with a high pH as they exhibit superparamagnetic behavior in alkaline medium. In vitro studies with PLGA nanoparticles have shown favorable activity in both strongly acidic as well as strongly alkaline medium. The surrounding pH of the chitosan nanoparticles also causes changes in its outer structure and modifies its surface. Studies show that the therapeutic effect of chitosan nanoparticles is changed by the acidic pH present around the tumor cells (Nguyen and Lee 2017). Additionally, the antimicrobial and antioxidant behavior of chitosan nanoparticles also depends on the pH of their surrounding medium. Studies also demonstrate that pH is important during dendrimer-mediated drug delivery where both the interior and exterior surfaces of dendrimers can be influenced by the surrounding pH, which ultimately affects the loading of various drugs (Maiti et al. 2005).

3.1.6 Temperature

The stable synthesis of nanomaterials is strongly dependent on the temperature of synthesis. Typically, gold nanomaterials are synthesized at high temperatures. Not only that, the plasmonic behavior of gold nanomaterials is well utilized in photothermal therapy where local heat is generated by light-induced excitation of these nanomaterials. As the gold nanomaterials start resonating, and upon an increase in temperature, gold nanomaterials burst out, releasing the drug on site (Vines et al. 2019). The formation of polymeric micelles and their shape and size are also highly dependent on temperature. A slight change in temperature can change the CMC value during the micelle formation, leading to a change in their size or shape. Likewise, at high temperatures, gelatin nanoparticles tend to loosen its structure by becoming flexible. This thermosensitive behavior of gelatin nanoparticles assists in realizing thermo-responsive drug delivery. Temperature is also an important factor in determining the fate of PLA nanoparticles. High temperatures favor the degradation process of PLA nanoparticles.

3.2 Interaction Mechanism

Several classifications of nanomaterials have been discussed in the above sections. The interaction of nanomaterials with biomolecules occurs through a wide-angle point-of-view. Nanomaterials interact with biomolecules in several biomedical applications, such as diagnostic tools like biosensors, bioimaging tools, nanocarriers, targeted drug delivery, etc.

The most pertinent biomolecules that interact with the surface of nanomaterials are nucleic acids and proteins. Binding with proteins can be via specific and

non-specific adhesion as proteins have many contrasting binding sites due to post-translational changes and key amino acid structures. Adding to that, immune response to the biocompatibility of nanomaterials is critical in proteins. The highly specific base pairing, good physicochemical and mechanical stability, and ease of accessibility of nucleic acids result in an appropriate receptor for biomolecular nano-assembly.

Typically, during the nanomaterial interaction with physiological biomolecules: (a) the structure and intended purpose is compromised once the chosen nanomaterial is surrounded by other dormant biomolecules. Hence, custom-made nanomaterials play a vital role here. They are highly specific to their target biomolecules; (b) nanomaterials entering different pathways in human anatomy are significantly influenced by their force of interaction. For instance, inhaled nanomaterials will directly interact with and affect the pulmonary system of the body. Based on these interactions, two major methods of immobilization are studied through interactions with various kinds of biomolecules, namely, physical and chemical (Fig. 3).

3.2.1 Physical Immobilization

Adsorption

This method involves the physical binding of the biomolecule to the surface of the nanomaterial. The nano-moiety can be either organic or inorganic. Binding occurs with the help of hydrogen bonds or Van der Waal forces. This method is widely used for the attachment of nucleic acids and binding proteins, avidin-biotin, hormones, and receptors as it is a simple and economic method of immobilization with minimal loss of activity. However, it provides a lower surface area for the binding to occur,

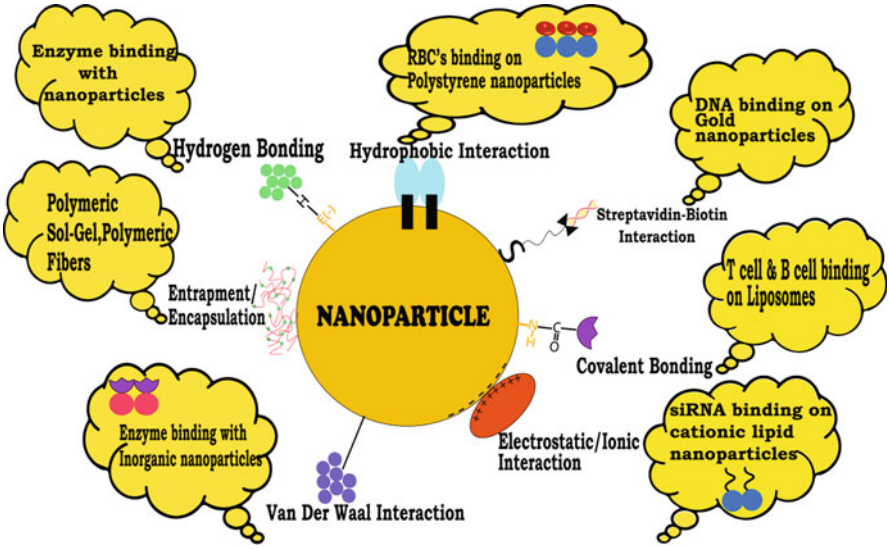

Fig. 3 Types of interaction between nanoparticles and biomolecules

and the yield is also low due to desorption and inactivation of the material. For example, polystyrene nanoparticles get attached on red blood cells (RBCs) by hydrophobic interactions, and various enzymes bind with inorganic nanoparticles through Van Der Waal as well as hydrogen bonding.

Similarly, adsorption of oppositely charged biomolecules on nanoparticles such as negatively charged siRNA on cationic lipid nanoparticles is also observed by electrostatic/ionic interactions. Binding of β-lactoglobulin on silicon nanoparticles with negative charge also occurs through ionic interaction.

Entrapment/Encapsulation

In this method of immobilization, biomolecules are not directly attached to the nanomaterial; they are attached with the help of a polymer matrix. They are attached with the help of gels or fibers, which are in turn attached to the nanomaterials. A variety of materials can be used for this purpose, like sol-gels, polymers or polymer sol-gel conjugates, and many other inorganic materials. Generally, chemical modification of the molecule is not required during this process, leading to the formation of more stable structures. However, the biomolecule may leak from the matrix formed. Another widely used physical interaction is the streptavidin-biotin complex. Interaction using streptavidin-biotin is considered as the strongest non-covalent interaction. It helps in binding DNA onto gold nanoparticles for DNA delivery applications. Additionally, it is also seen that human embryonic kidney cells form a streptavidin-biotin interaction with PLA-PEG nanoparticles.

3.2.2 Chemical Immobilization

Covalent Binding

In this method of immobilization, the biomolecules are bound to the surfaces of nanomaterials with the help of functional groups that form covalent bonds between them. Different types of organic and inorganic groups like phenolic groups, carboxyl groups, etc., are used for this purpose. Covalent binding is very strong and leaves no scope for the leakage of the biomolecule. For example, T cells and B cells containing free thiol groups on their surfaces bind covalently onto nanoparticles like liposomes. However, these bonds can alter the activity of the biomolecules.

Cross-Linking

Cross-linking involves direct binding of the biomolecules to the surface of the nanomaterials with or without the use of support structures. This method is generally used for water-insoluble molecules. The most commonly used reagent for this purpose is glutaraldehyde. Cross-linking is highly stable and there is little chance of the structure getting distorted. However, this method is costly and can cause changes in the active site of the nanomaterial.

3.2.3 Generation of Reactive Oxygen Species on Nanoparticle-Cell Interaction

Nanoparticles, besides self-oxidation, also produce oxidant effects by initiating the generation of reactive oxygen species (ROS). Highly reactive surface, redox surface reaction in metallic nanoparticles, and nanoparticle interaction with cells are the main factors responsible for the generation of ROS during the use of nanoparticles in biological applications. Besides, physiochemical properties of nanomaterials initiate the production of ROS on interaction with phagocytic cells. Typically, the smaller sized nanoparticles are responsible for more ROS production due to their highly reactive surface. The amount of ROS generated by nanoparticles directly affects their toxicity extent. Many nanoparticles on internalization in the cells reach the mitochondria and depolarize its membrane, which leads to abnormal electron transport, stimulating the production of ROS. The depolarization of the mitochondrial membrane is caused due to damage of phospholipids by the ROS present in the mitochondria. Many nanoparticles such as zinc and copper show this mechanism on interaction with human cells. One such study showed that when nanoparticles accumulated in the lungs, inflammatory cells were activated, which led to the generation of ROS, thus damaging the lung cells (Manke et al. 2013).

4 Compatibility of Nanomaterials in Biological Systems

It is inevitable that the design of nanomaterials, including its shape, size, surface modification, plays a crucial role in optimizing their favorable interactions in each biological system. Studies have demonstrated that small nanoparticles of size less than 10 nm can enter the human cellular system. Therefore, it needs a critical understanding to control the concentration of these nanomaterials that keeps the cytotoxicity level under non-adverse limits. Several studies are being conducted to evaluate the possible biocompatibility and toxicity of these nanomaterials in any biological microenvironments and to engineer biologically safe nanomaterials which may be of potential use in biomedical applications.

4.1 Inorganic Nanomaterials

With the rapidly increasing use of nanomaterials in biological applications, utilization of metallic nanoparticles is also increasing at a very fast rate. Due to their unique innate physiochemical properties, metallic nanoparticles are being widely used in in vitro applications such as bioimaging and biosensing. Despite several prospective applications, concern about the toxicity of inorganic nanomaterials is still a major concern. The toxicity of inorganic nanomaterials refers to the side effects caused by the nanoparticles to its surrounding medium during their activity. Because the cause and extent of toxicity exhibited by nanoparticles vary with shape, size, and concentration, toxicological studies for each nanomaterial are needed to be carried out before their actual use in in vivo conditions. Au nanoparticles are currently the most

explored nanomaterial for use in the medical field, though it is seen that gold nanoparticles with a size below 4 nm show great toxicity by penetrating inside the cell and binding with DNA (Kamiar et al. 2013). A study showed that accumulation of Au nanoparticles in the liver and spleen regions of rat models ultimately causes alteration in their gene expression (Kamiar et al. 2013). It has been reported that Cu nanoparticles when used in a high concentration of more than 100 μM remain accumulated in human cells for a long time, which may damage the cell membrane and induce oxidative stress, which ultimately leads to cell death. Toxicity caused by Cu nanoparticles is primarily due to the generation of reactive oxygen species. Moreover, their tendency to oxidize limits their storage in a vacuum-sealed, dry, and cool environment. On the other hand, Se nanoparticles are less toxic in nature and the toxicity is dose dependent. Iron oxide nanoparticles, which are frequently used in imaging and drug delivery, show different biochemical reactions depending on their surface chemistry. Their toxicity effect is shown by the generation of a high amount of reactive oxygen species, which results in cell injury. Due to their nontoxic nature, ZnO nanoparticles are widely used in cosmetics; moreover, cosmetics containing ZnO nanoparticles do not penetrate deeply into the skin. Among the various inorganic nanomaterials, the highest level of toxicity is shown by quantum dots. Quantum dots can easily get excited and cause harm to human body, and their oxidative nature directly causes damage to DNA; thus, quantum dots are most preferred in in vitro applications like biosensing and bioimaging. Despite the toxicity issues, inorganic nanomaterials possess overwhelming advantages for use in the medical field. For this purpose, many surface modifications are carried out to improve the biocompatibility of the inorganic nanomaterials. Often, biopolymers are coated on these nanoparticles, which prevent unnecessary interaction between cells and inorganic nanoparticles, and green synthesis methods are adopted for these nanoparticles, which can inherently make them biocompatible in nature and decrease their toxic effect.

4.2 Organic Nanomaterials

Organic nanomaterials, either natural or synthetic, are highly biocompatible and biodegradable in nature. These are the least toxic nanomaterials reported till date. However, in some situations, organic nanomaterials can also show a low amount of toxicity which is caused as a result of changes in the molecular structure of the material or high surface energy which makes them highly active to interact with the cells and other biomolecules inside the human body. For instance, cellulose nanofibers were seen to cause inhalation toxicity in rats. Similarly, when chitosan nanoparticles are taken through the nasal pathway, pro-inflammatory responses are triggered. Different pathways of taking organic nanoparticles can cause different effects. A study showed that chitosan nanoparticles can enter the brain and their accumulation may lead to apoptosis of neurons and inflammatory response (Huang et al. 2005). Typically, by taking care of organic nanoparticles during their formation and preventing accumulation in any part of the body, the current low amount of

toxicity exhibited by these nanomaterials can also be prevented, making organic nanomaterials promising materials for biomedical applications.

5 Conclusions

Nanotechnology has a remarkable influence in the field of biomedical field. The nanomaterials used for the application in this field have a direct impact on our lives. Thus, to get a clear insight, in this chapter, we have classified the nanomaterials and extensively discussed their characteristic features and applications. Further, we focused on the interaction mechanism of nanomaterials with various biomolecules and factors affecting this interaction, which is a key point to be taken care of while using nanomaterials in medicine. Finally, the compatibility in terms of stability and cytotoxicity is described in detail.

The various inorganic and organic nanomaterials used for different biomedical applications have their own unique features as well as limitations. Inorganic nanomaterials such as gold, silver, copper, etc., are easy to synthesize with the possibility of alterations in properties according to one's use of interest. However, organic nanomaterials such as liposomes, micelles have a definite set of properties which cannot be altered. On the other hand, polymeric (organic) nanoparticles find an upper hand in biocompatibility as compared to metal and metal oxide nanomaterials. Polymeric nanomaterials such as liposomes, starch, dendrimers, micelles, etc., show very less cytotoxicity because of their easy biodegradability to simpler non-toxic molecules. Typically, polymeric nanoparticles are more widely used in in vivo applications of drug and gene delivery, preparing scaffolds for implants as they can easily interact with cellular components, whereas inorganic nanoparticles are applied more in in vitro techniques such as bioimaging techniques as contrast agents. Many nanomaterials approved by the US FDA are already being used in clinical practices. Some of these include Lipofectin – a liposomal formulation being used for DNA transfer; Genoxal – a polymeric micelle used in the treatment of breast cancer; ZnO nanoparticles incorporated in various cosmetic products; silver nanoparticles used in bone implants; gold nanoparticles utilized in the treatment of various types of cancer using photothermal technology; etc.

Despite so many accomplishments till date, it is evident that we are still waiting to cross the tunnel and fully utilize the promising potential of nanomaterials. A deeper understanding of toxicity related to nanomaterials and practices to overcome this issue need to be worked upon. Alongside, further studies related to the interaction of nanomaterials inside biological systems will result in more efficient and safer biomedical applications.

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Review on IPR and Technological Advancements in Nanotechnology for Nanomedicine

Ravi Gor, Saurav Panicker, and Satish Ramalingam

Contents

Abstract

Nanotechnology is the branch of science which deals with particles of dimensions lesser than 100 nm in one dimension. In this field of study, identification, characterization, and its application, etc., of the nanomaterial are explored. This enables the understanding of nanomaterials and possible applications in the field of electrical, electronics, biomedical, nanomedicine, molecular biology detection, nano-device development, etc. The advent of nanotechnology has led to the invention and/or discovery of a variety of nanomaterials. In this chapter, we have summarized nanomaterials and their application in the nanomedicine field. We have focused on the compatibility of the nanomaterials with the biological system. The chapter further deals with engineering a nanomaterial for efficacious targeted drug delivery in myriad diseases like cancer, Alzheimer's disease, and many more. Furthermore, the chapter discusses the nanomaterial application in the field of growth-dependent marker expression, faster and accurate cell analysis

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_20-2

using nanomaterial technology. In the second part of this chapter, we look into the bioethics and biosafety issues while handling mammalian cells. This part focuses on understanding the impact of the experiment on the environment and the researcher. It further forms the guidelines for experimentations and the requisite code of conduct for researchers in keeping the environment and health of the researcher safe. The final part focuses on the importance of intellectual property rights (IPR) on an industrial and academic scale.

Keywords

Nanomedicine · Nanotechnology · Intellectual Property Rights · Biomedical

1 Introduction

Nanotechnology is the scientific field that focuses on the production of materials and devices at the nanoscale. A nanoscale or a nanometer is a ten to the power negative ninth of a meter. Things as small as that of nanoscale are made to achieve the goals of futuristic machines, technology, etc. The application of nanotechnology in the medical field opens up a new horizon of treating existing and novel diseases. The major advantages of the nanomaterial are their smaller size and larger surface-to-volume ratio. A reduction in the size of the drug formulation increases the dissolution of the material directly (Liversidge and Cundy 1995). This is due to the higher availability of the formulated compound in the small area as compared to a low number in the same area, increasing the solubility of a compound at the release site. This also helps in increasing the bioavailability of the drug compound. Nanotechnology has shown promising application in the various fields of health care like imaging, diagnostics, drug delivery, therapeutics, etc. (Murphy et al. 2015). Nanomaterials are engineered as per the specific applications to serve a specific purpose such as targeted drug therapy for different diseases, in the biomedical and bioengineering field.

Nanomaterials can be categorized into four different types based on the materials they are made up of. First, carbon-based nanomaterials, which are comprised mostly of carbon, can encompass hollow sphere, ellipsoids, or tubes. Fullerenes are spherical and ellipsoidal, whereas nanotubes are cylindrical. Secondly, the metal-based nanomaterials include metals like gold, silver, metal oxides, etc. Third, dendrimers are nanopolymers build from branched units. Dendrimers contain interior cavities into which cargo can be placed and thus widely used in drug delivery applications. Fourth are the composites, and these are the combinations of two or more nanoparticles, as illustrated in Table 1 and Fig. 1. These are made or used to have a more efficient nanomaterial for the application by complementing the pros and cons of the different materials.

Intellectual property rights (IPR) is a list of various protection acts and rights that are detailed with considering a relevant international law such as: the Paris Convention for the Protection of Industrial Property (1883), the Madrid Agreement

Table 1 Types of nanomaterials and their examples

Sr. No.	Nanomaterial type	Example	Shape	Size	Reference
1	Carbon based	Single-walled carbon nanotubes	Cylindrical	Diameter – 1 nm	Husen and Siddiqi (2014), Li et al. (2019)
		Multi-walled carbon nanotubes	Cylindrical	Diameter – >1 nm (based on the number of walls)	Husen and Siddiqi (2014), Li et al. (2019)
		Fullerenes	Spherical	C28, C36, and C60	Husen and Siddiqi (2014)
		Graphene	Hexagonal crystal lattice		Husen and Siddiqi (2014), Li et al. (2019)
2	Metal based	Gold, silver, and metal oxides	Cylindrical, spherical, etc.	Diameter – 25–40 nm and < 100 nm (some metals)	Barik et al. (2021)
3	Dendrimers based	Poly (amidoamine)	Spherical	Diameter – <100 nm	
4	Composites based	Carboxymethyl guar gum/Ag	In the form of gel, sheets, etc.	Diameter – <100 nm	Grover (2020)

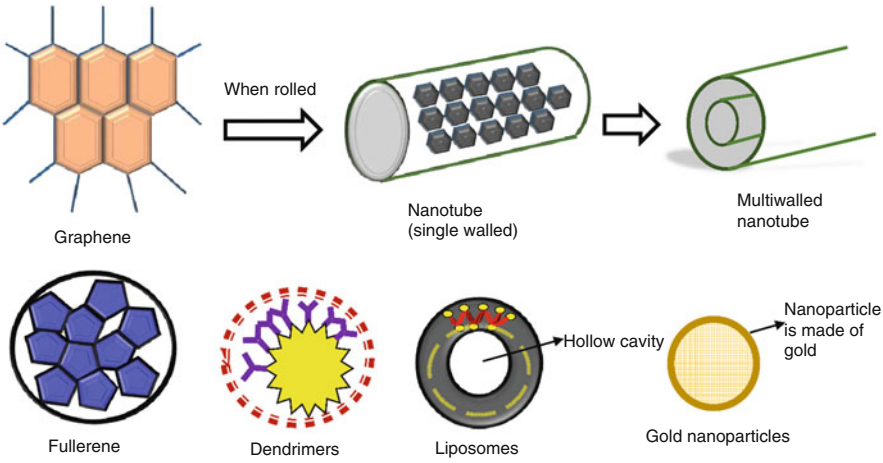

Fig. 1 Types of nanomaterials

Concerning the International Registration Marks (2891), and the Protocol Relating to that Agreement (1989), etc. (Morcos and Khneisser 2020). In general terms, the

IPR protects the trademarks, copyright, patents, industrial design, etc., for the inventor nationally and/or internationally.

2 Compatibility of Nanomaterials Towards Biological Interactions

There is a discrepancy in defining a nanomaterial among different scientists, although enthusiastic contributors have worked out an acceptable definition based on its size. It states that a nanomaterial must have a single unit size of one dimension less than 100 nm. The nanoscale size of the material makes it useful to create a vesicle or a cargo that can carry the drug for the treatment. Not all nanomaterials can be used in the biological field due to their toxicity, complexity, etc. For the nanomaterials to work in the medical drug delivery system, they should be compatible with the human environment like gut, bloodstream, extracellular matrix, etc. Nanomaterials can be divided based on their origin, i.e., either naturally available (chitosan) or man-made or also called engineered in labs (carbon nanotubes).

From the establishment of nanomedicine, endless fabrication of novel nanomaterials has led to the selection process of the nanomaterials for application in the medical field. This has led to the big question regarding the biosafety and compatibility of the newly made nanomaterials towards the biological system. When used in the medical diagnostic field, the nanomaterial interacts with the cells, proteins, membranes, biomolecules, etc. This interaction solely depends on the Brownian motions and other physical motions and includes various biological interactions. The nanomaterial interacts with the biological system at the nanoscale in various ways like with protein, interacting with the ligand and internalization in the cell, and other interactions in the cell. Nanomaterials are relatively larger and similar to the size of the neighboring protein molecules. Once inside the bloodstream, the nanomaterial reacts *via* interacting with the freely available proteins and antibodies, which will lead to the agglutination of the nanomaterial before reaching the target site (Zhang et al. 2012). The surface of the nanoparticle makes a big impact on its random or targeted interaction with the available protein molecules and cells. These nonspecific interactions of the nanomaterials can be reduced significantly *via* a change in the surface, use of polyethylene glycol (PEG), etc., for the nanomaterial being developed (Bertrand and Leroux 2012*;* Owens and Peppas 2006). The protein-nanomaterial interaction is also used to deliberately channelling the nanomaterial to the target site for drug release. The downside of the nanoparticle-protein interactions is the immunogenic reaction against the nanoparticles. The first line of defense, the complement system, and the associated proteins target the nanoparticles and try to neutralize them. This is mainly due to the physicochemical surface properties of the nanomaterial, which triggers different pathways of complement cascade activation. As an example, the classical pathway is activated through the interactions with specific proteins like antibodies, etc. Surface recognition by the mannose-binding lectin (MBL) triggers the lectin pathway, through pathogen-associated motifs. The presence of factor B in plasma gets deposited on the nanomaterial and further leads

to the C5 convertases that cleave C5 and lead to the deposition of the terminal membrane attack complex which can lyse pathogens and senescent cells, and releasing proinflammatory mediators (Zhang et al. 2012). After making the way out safely with the bloodstream without any major interaction with the blood proteins, next is the interaction of the nanomaterials with the target site *via* a specific ligand molecule or according to the pH drug release occurs, etc. Nanomaterials are coated with various biomolecules like proteins, lipids, etc. This formation of a nanomaterial is called a "bio-corona" formation (Bhattacharya et al. 2016). A cell-surface ligand works like a homing signal for the engineered nanomaterial to target and release the drug at the target site. This interaction can be widely used for concentrating the drug to the target site of the disease *in vivo* and *in vitro* to detect a specific biomarker for a particular disease (Farokhzad and Langer 2009; Ferrari 2005). Nanomaterials decorated with specific ligand molecules will also help in the endocytosis by the cell and drugs can be released more efficiently (Gao et al. 2005). Once the nanomaterial is internalized *via* endocytosis, its fate purely depends on the endocytosis pathway and the physiochemical properties of the nanomaterial. Based on which receptor is used for the endocytosis of the nanomaterial, it will either go for the lysosomal degradation (clathrin-dependent receptor-dependent) or endosomal accumulation and non-degradative pathway of sorting in the cell (clathrin-dependent receptor-independent) (Bareford and Swaan 2007).

The carbon-based nanomaterials have also shown similar interactions with the biological system. Carbon-based nanomaterials including fullerenes, nanodiamonds, single- and multi-walled carbon nanotubes, and graphene oxide are the potential nominees for medical applications. Carbon nanotubes (CNTs) have strong optical absorption in the near-infrared, Raman scattering, as well as photo-acoustic properties; this increases the implication of the CNTs *in vivo* applications. Fullerenes and nanodiamonds are also shown important in the cancer medicine area. Another carbon-based nanomaterial, graphene shows a promising application in drug delivery because of the large surface area and easy functionalization opportunities. Further, its mechanical properties open up huge applications in tissue engineering and regenerative medicine (Zhang et al. 2012). The bio-coronation of the carbon-based nanomaterials had a huge impact on the fate of the nanomaterial in cellular degradation and biodegradation. The interactions that occur at the small molecule, ion adsorption, nucleic acid, lipid, protein, oxidative reactions, and biological degradation are some of the interactions of graphene a carbon-based nanomaterial with biological interactions. Graphene has a high surface area and thus it is a potent sorbent for a variety of small-molecule solutes in physiological fluids. The major biological consequence is the depletion of micronutrients. It can be used as an artifact in dye-based assays (Wörle-Knirsch et al. 2006) and shows an ability to carry small molecule drug cargoes (Wörle-Knirsch et al. 2006). Carbon-based nanomaterials have also shown an interesting interaction with nucleic acids. Graphene oxide (GO) is reported as a protective nanomaterial against enzymatic degradation (Wörle-Knirsch et al. 2006)$^{-}$(Wang et al. 2010). GO has also shown selective adsorption of the ssDNA against the dsDNA (Lu et al. 2010; Ren et al. 2010; Wang et al. 2010; Xu et al. 2010b). Further, it is found that the interaction of

negative charges on DNA and GO, and further the lower pH and the high ionic strength support the adsorption of the small oligomers (Wu et al. 2011). Interaction with lipid and proteins, the graphene monolayers and multiple of them were easily able to localize in the hydrophobic core with a minimum agitation of the overall bilayer only when the layer number is small. Protein and amino acid adsorption on the nanomaterial surface is also observed. The surface of carbon nanomaterial reacts with the antioxidant glutathione and reported its activity for the glutathione-O_2 reaction (Zhang et al. 2012). The key characteristic of carbon-based nanomaterials is the resistance to biological degradation. This increases the biological durability and the bio-persistence in intact organisms. Further, this also raises concerns regarding the decomposition or removal from the organism's system. Graphene oxide and carboxylated single-walled nanotubes are susceptible to oxidative attack by hydrogen peroxide and horseradish peroxidase (Allen et al. 2008; Kotchey et al. 2011).

The abovementioned interactions of nanomaterials are propagated irrespective of their natural or man-made origin, like carbon-based nanomaterials. These findings will help in designing safer and more effective nanomaterials for biological purposes in the future.

3 The Precision of Targeted Drug Delivery Using Nanocarriers

The conventional method of drug treatment is based on majorly two ways oral and intravenous injection. This will make the drug to be modified in such a way to sustain the different environment of oral cavities until it is absorbed, and reaches the target site and performs its action. The disadvantages of these are that the structural modification could hamper the efficiency of the drug, reduce the concentration of the drug when compared to the intake, the cancer drugs are produced such as to target the rapidly dividing cells, which make collateral damage for the host by killing the fast-growing cells like hair, etc.

To overcome these disadvantages of the conventional drug delivery system, nanocarriers are produced and engineered in such a way to target diseased tissue such as cancer tissue. Nanocarriers can be defined as a nanomaterial used to transport cargo like a drug to the infected individual. Further, when we engineer the nanomaterial to target the specific cells of the body, it is termed as a targeted drug delivery using nanocarriers. The aim of using a targeted drug delivery system is to concentrate the drug release in the targeted site, reducing the side effects of the drugs to the normal tissues.

Drug carriers at the nanoscale can be synthesized from different types of materials, including inorganic nanoparticles, polymer nanoparticles, and a combination of both. Targeted drug delivery can be made *via* two approaches: active and passive targeting. It helps in homing the nanoparticle to the target site of the system. Actively targeting nanomaterials utilizes a surface modification of the nanomaterials also known as drug carriers. These modifications include the inclusion of the ligands such as aptamers, small molecules, antibodies, peptides, glucose, and antibodies that

bind to the targeted cell/tissue of interest. Further, the drug delivery is carried out *via* the ligand-mediated internalization with the target cell. Normally the binding targets of the modified nanocarriers include differentially overexpressed receptors/antigens on the plasma membrane of the target cells.

Whereas on the other hand, the passively targeting method works by the inherited bio-physiochemical properties of the nanoparticles which include size, shape, charge, and flexibility, etc. This does not involve any specialized modification of the nanomaterial for the treatment. Although the optimization of size, shape ratios and using positively charged nanomaterial to better interact with the negatively charged cellular membranes. The diagnostic data will help in the determination of whether the treatment will be susceptible for the patient or not. Also, the universally designed nanomaterial will be beneficial for the patient for the treatment or not. *In vivo* studies in rodents for breast cancer have shown that the passive targeting approaches can be used for the targeted drug delivery treatment. Further, the targeted drug delivery can be used to target the intracellular organelles directly. The genetic disease related to the mitochondrial genome defects can be treated with the delivery of the direct DNA into the mitochondrial matrix. Nanoparticles wrapped with macrophage or leukocytes membranes recognize tumors. Also, hybrid membranes such as erythrocyte cancer cell hybrids can further increase the specificity of the nanoparticles. With these modifications, the drug activity has been increased two- to threefold (Zhang et al. 2012). The property of the material used can also help in the distribution of the nanomaterials. A poly(B-amino-ester) (PBAE) terpolymer/PEG lipid conjugate was optimized for the lung localization, which is twofold more than the pre-optimized from *in vivo* and *in vitro* (Kaczmarek et al. 2018). Other types of PBAE polymers are developed to target glioblastoma cells over healthy cells *in vitro* (Karlsson et al. 2019).

The use of nanomaterial and their different variants has opened up a huge avenue for delivering the drug compound to the target site either actively or passively. The drug delivery can also be made cellular organelles specific in certain cases with the use of specific nanomaterials. Further, the bio-physiochemical properties of the nanomaterials can be engineered such that the nanomaterial can be used to target precisely the diseased tissue or cell. In the field of precision medicine, relevant application of drug delivery to the diseased cell or tissue, the usage of nanomaterial has allowed improved cellular targeting, relatively less off-target effects, and more personalized therapies to the patients. All of this can be achieved by understanding the nanomaterial, its biological interactions, increasing the accumulation of the drug compound at the site of interest, and susceptibility for on-demand drug release (Mitchell et al. 2021).

4 Enhanced Sensitivity for Ascertaining Growth-Dependent Marker Expression

Molecular markers are the alterations in the gene sequences, expression levels, and protein structure the function of proteins. These observations and their relative expressions are used to detect the cancers at an early stage, and determine the prognosis, and monitor disease progression or therapeutic response of cancer treatment (Sidransky 2002). Similarly, certain genes are expressed in the initial stages of life, and then their expression is inhibited in the later stage of development. This shows the importance of the expression of individual genes in the normal development of an individual. The product of the gene in the form of a protein, receptor, expression of genes, etc., is called a marker for a particular phenotype of the cell at that particular stage. As an example discovery of the biomarkers for colorectal cancer involves the tumor endothelial markers (TEM), TEM1, TEM5, TEM7, and TEM8 (Pietrzyk 2016). The molecular and computational methods used for the detection of the marker for a particular phenotype of the cell are serial analysis of gene expression (SAGE), LC-MS/MS, gene ontology and network analysis, next generation sequencing, digital PCR, measurement of enzymatic activity and two-dimensional gel analysis of samples, antibody-based detection methods, etc. (Pietrzyk 2016; Sidransky 2002; Xu et al. 2010a).

The experimental technique called serial analysis of gene expression (SAGE) is applied to gain a direct and quantitative measure of gene expression. The principle behind this technique is the isolation of unique sequence tags from an individual mRNA and concatenation of tags serially into long DNA molecules for lump-sum sequencing. Certain drawbacks encourage the use of nanomaterials so as to reduce the background and get a clear image of the data. The PCR step produces linker dimers, and to minimize it, biotinylated PCR primers were introduced. This resulted in the biotinylated ditag products at an early stage in the SAGE protocol, which allows the removal of the linkers by binding to streptavidin beads used at a later stage. This adaptation along with others in the protocol has enhanced the clarity of the results for a better analysis (Yamamoto et al. 2001).

Along with the SAGE, a polymerase chain reaction (PCR) technique is a widely used technique to understand the marker expression at the different stages of cellular development. PCR is an error-prone process due to its working *in vitro* and results in the reduced specificity, fidelity, and efficiency of the PCR reaction. The introduction of nanotechnology has made a significant improvement in the technique. This new type of PCR reaction setup is called nanomaterial-assisted PCR or also known as nano-PCR. It is a new era of improved PCR reactions using gold nanoparticles (AuNPs), graphene oxide (GO), quantum dots (QDs), reduced graphene oxide (rGO), upconversion nanoparticles (UCNPs), carbon nanotubes (CNTs), fullerenes (C_{60}), and other metallic nanoparticles and nanocomposites (Tong et al. 2012) are used. These nanomaterials in the field of PCR have increased the yield and specificity of the target product which reduces the background noise of the results and further better result analysis of the marker expressions (Yuce et al. 2014).

The study of the markers for the identification of a particular disease or involvement of a protein at a specific stage in the growth and development of an individual cell is very important to decipher the functioning of a cell. The incorporation of the nanomaterials in the existing techniques, assays have improvised the analysis and detection of the markers responsible for the growth of the cells.

5 Nanomaterial-Driven Faster and More Accurate Cell Analysis

As we all know, the cell is the basic fundamental unit of life. The cell is vital in steering the ever-crucial biological processes such as DNA replication, DNA repair, transcription, protein synthesis, protein trafficking, metabolism, and cell signaling (Wang et al. 2014). The pathogenesis of myriad diseases has been linked to anomalies in cellular compartments that partake in various biological processes. Hence, there arises an imminent demand for technological advancements which foster monitoring of the cell at higher resolutions. Nanotechnology offers us cutting-edge modalities at the nanoscale level for explicating the complex biological systems within the cell (Nagamune 2017; Salata 2004). Integrative input of nanotechnology in biotechnology has garnered profound prominence in circumventing major limitations in present-age diagnostics and therapeutics (Qi et al. 2019; Yong et al. 2009; Zhang et al. 2008). Emphatically, nanotechnology has revolutionized the fields of pharmacology (Qi et al. 2019) and medicine (Wang et al. 2014) in the past decade. Employing nanotechnology in precision medicine (De Matteis et al. 2017; Mitchell et al. 2021; Qi et al. 2019) shows the future trend in its medical applications for augmenting patient-specific benefits in direct clinical settings (Mitchell et al. 2021).

Nanomaterials are the basic key constituents of nanotechnology. Nanomaterials are below 100 nm in size, in at least one dimension. Concerning their dimensionality, nanomaterials can be categorized into many types (Kolahalam et al. 2019). The types of nanomaterials based on their dimensionality are as follows:

1. Zero dimensional: nanoparticles, quantum dots
2. One dimensional: nanorods, nanotubes, and nanowires
3. Two dimensional: nanofilms, nanolayers, and nano-coatings
4. Three dimensional: nanocomposites, nanowire bundles

Nanomaterials are further differentiated based on the components with which it made; the frequent integral elements that constitute a nanomaterial being carbon, semiconductors, and metals.

Nanomaterials possess certain novel specifications such as enhanced bio-permeation, (Nakamura and Watano 2018), improved biocompatibility (Yong et al. 2009), huge surface area to mass ratio (Adabi et al. 2017; Zhang et al. 2008), and higher sensitivity and superior photostability (Yong et al. 2009) which can be remarkably exploited for exalted precision in cell analysis, especially single-cell detection (Wang et al. 2014). The unique capability of nanomaterials to tailor their

abovementioned characteristic properties at the molecular scale furnishes us with a plethora of prospects to explore biomolecular interactions at the cellular resolution (Contera et al. 2021). Salient cellular attributes such as cellular morphology, elasticity, cellular thermal gradients, ionic equilibrium, and structural fidelity of biomolecules (Wang et al. 2014) can all be extensively scrutinized because of the recent scientific expansions in the application of nanomaterials (Wang et al. 2014). Some of these scientific escalations in the biotechnological implementation of nanomaterials in the arena of cell analysis will be the prime objective of our discussion.

Nanomaterials have been used in optical microscopy (Zheng and Li 2012). For delineating the cell membranes, near-field scanning optical microscopy (NSOM) is used (Zheng and Li 2012). The novel breakthrough of NSOM lies in the fact that NSOM has surpassed the diffraction limits, providing an in-depth resolution of lesser than 10 nm, making it valuable for nanoscale cellular imaging (Zheng and Li 2012). NSOM has been employed in the distribution and localization of proteins and lipids, especially integrins (Van Zanten et al. 2009; Zheng and Li 2012). But NSOM has two major drawbacks, the first being its lag in the scanning process, while the second being its reduced penetration depth (Zheng and Li 2012), hence making it unsuitable for investigating dynamic biological processes within the cell (Zheng and Li 2012). To troubleshoot the above problem, optical fiber-based nanosensors have been developed (Zheng and Li 2012). The optical fiber-based nanosensors have been further upgraded for evaluating calcium concentrations, cancer marker expression levels, metabolite concentrations, and instantaneous variations of biochemicals within cells. (Zheng and Li 2012) Surface-enhanced Raman spectroscopy (SERS) active nanoprobes is another nanoscale mapping technique to investigate the molecular interactions within the cell (Zheng and Li 2012). SERS nanoprobes are way better than fluorescent-based detection in terms of sensitivity (Zheng and Li 2012). SERS nanoprobes have been utilized in appraising the pH in live human cells (Zheng and Li 2012). Quantum dots developed from semiconductor materials are made use in protein labelling, which is, labelling of microtubules, actin, and even chromosomes (Wang et al. 2014). The biggest advantage of quantum dots is that QDs have a higher fluorescence lifetime and longer stability (Wang et al. 2014).

Scanning electrochemical microscopy (SECM) makes use of nanoelectrodes to comprehend the electrochemical fluctuations in cells (Wang et al. 2014). SECMs have direct applications in the nanoscale mapping of electrochemical gradients in breast cells (Zheng and Li 2012). A nanoelectrode is so minuscule that its diameter is 1/1000th of a cell, and hence can be used to compute membrane potential and subcellular redox values, without damaging the cell membrane (Zheng and Li 2012). SECMs have also been utilized in studying membrane permeability and appraising enzyme actions (Wang et al. 2014). The implementation of silicon nanowire-based field-effect transistors (FET) for measuring bioelectricity in neurons is a novel application of nanotechnology in neurochemistry (Zheng and Li 2012). Owing to their extremely small diameter of less than 1 nm, single-walled carbon nanotubes (SWCNTs) can interact with cells at a deeper spatial resolution and hence are employed in elucidating phagocytosis in real time in macrophages (Zheng and Li 2012). Scanning ion conductance microscopy (SICMS), in particularly Hopping

mode SICM (HPICM), is operated for capturing pictures of complicated and generally impenetrable structures, such as the auditory stereocilia. Even ionic gradient fluctuations and ionic signaling in both microvilli and cardiomyocytes are computed with sharp accuracy using SICM based patch-clamp probes (Zheng and Li 2012). Atomic force microscopy (AFM) is probably the best device in examining cellular processes. AFM is operated for scrutinizing ligand-receptor binding (Zheng and Li 2012). Peptidoglycan in prokaryotes was deciphered using AFM (Zheng and Li 2012). The exceptional feature of AFM is that it can compute the mechanical forces as minute as piconewtons, and therefore, AFM is widely used for decoding the mechanical characteristics of any cell, including distinguishing the differences in mechanical properties between tumor cells and normal healthy cells (Zheng and Li 2012).

Nano-thermocouples have been developed in assessing intracellular thermal gradients in single cells at a nanoscale level (Wang et al. 2014). These provide great opportunities to invent cellular-level biomolecular sensor (Wang et al. 2014). NanoSIMS is a nanoscale spectrometry approach applied in the identification of microelements constitution in cellular compartments and understanding calcium flux. Nanomanipulation devices such as optical tweezers are useful in controlling the existing microscopy techniques for single-cell manipulation. Combinatorial systems of nanomaterials promise higher efficacy to delineate cellular operations at the nanoscale level. Other promising nanomaterials include nano-gels, nano-labels for imaging, and nanocapillary electrophoresis for the identification of neurotransmitters from a single cell. Nanochannel electroporation can be exploited for more efficacious drug delivery and transfection of any biomolecules (Wang et al. 2014).

To recapitulate, we can hereby understand the broad breadth of nanotechnological applications in pursuit of answering questions related to biologics. The above-explained nanomaterials have been instrumental in obtaining vast data regarding the intricate biological circuits in cells. Predictably, nanomaterials-driven cell analysis will experience a rapid upsurge in the upcoming decade.

6 Reporting the Newly Identified Gene Sequences

It has been a couple of decades since we started understanding the cell and how it functions as an individual system and in the coordination of other cells to make a functional living multicellular organism. Cell's hereditary information in the form of DNA is purely responsible for regulating the healthy being of the cell. The sequence identity can be carried out by analyzing the existing database or generating reads and libraries from your experimental studies of interest. As an example, the early developmental stage of the *Homo sapiens* versus the later developmental stage. For locating or identifying the gene from the cell, the important requirement is the DNA sequence at a particular instance. The sequence can be whole genome, exon, RNA, etc., for the analysis of finding a novel gene sequence. Earlier, the finding was carried out *in vivo* by studying the behavior of an organism or isolating the DNA and studying it *in vitro*. The computer-based method of, also known as in silico analysis,

helps to make educated guesses about the location of genes by analyzing the sequence available. Various tools and algorithms can be used to reduce the noise from the sequencing data and identifying the new gene sequence from the analysis. The bioinformatician must make a call in understanding the data and performing a particular algorithm with a statistical method behind it to make an unbiased analysis of the reads and come to a conclusion of a novel gene sequence.

So basically, a novel gene can be identified using an old technique of observing the behavior of an organism and by isolating the gene and studying it further. A new method is to identify based on the analysis of the DNA sequence of the cell based on the known facts about the gene. A gene sequence starts with a start codon of methionine and ends with either of the stop codons, identifying the junction of the exons and combining it to form a complete gene sequence. RNA sequencing has opened up a new horizon for directly identifying the available RNA sequence at a particular time of a cell. This helps in identifying all the transcript variants of the gene. After identification of the gene, it can be reported in the publically available database like Gene Expression Omnibus (GEO), Gene Bank, Sequence Read Archive (SRA), etc. Here the reads are available freely online for any researcher to use and perform further analysis and conclude new findings from it.

7 Bioethics and Biosafety Issues in Handling Mammalian Cells

Bioethics is concerned with the ethical issues of performing advanced research in the field of biology and medicine. Whereas biosafety is concerned with the safety of environment and humans from the possible unfortunate effects from basic research, research and development, and also in the field of modern biotechnology. While using a mammalian cell for research purposes, it is equally important to follow the ethics and biosafety in the course time of research.

The biosafety recommendations are principally aimed at providing maximal protection of human health and the environment. Biosafety recognizes the importance of precautionary measures that will also directly benefit the quality of research activities involving animal cell cultures. The incidence of cross-contamination or inadvertent contamination of microorganisms and plaguing many researchers leads to unproductive and nonreproductive data. This also results in the misinterpretation of the results and a considerable waste of time, resources, energy, and funds (Herman and Pauwels 2015). Biosafety levels (BSL) are laboratory designations, which are based upon the degree of risk. There are four types of biosafety levels, and they are denoted as biosafety level 1 (BSL-1), biosafety level 2 (BSL-2), biosafety level 3 (BSL3), and biosafety level 4 (BSL-4), details are depicted in Table 2. BSL-1 in which open benchwork can be done via basic teaching or research laboratory often involves chemicals and reagents, which do not cause any disease in humans like *E. coli* (nonpathogenic strain) and yeast. It may not have a biosafety hood. BSL-2 uses to handle moderate-risk microorganisms responsible for infection and poses risk for infection *via* percutaneous or mucous membrane exposure. To contain the

Table 2 Deciding the right biosafety for the research sample being used

Biosafety	Risk level	Operations carried out	Impact on the user	Treatment availability	Examples
Biosafety level 1	Low	Nonpathogenic microorganisms	Less	Yes	Yeast, *E. coli*, etc.
Biosafety level 2	Moderate	Pathogenic microorganisms and animal samples	Moderate	Yes	*Staphylococcus*, cancer cell lines, etc.
Biosafety level 3	High	Samples with the known risk involved	High	Yes	*Mycobacterium tuberculosis,* etc.
Biosafety level 4	Very high	Samples with high-risk involved	Very high	No	Covid-19, etc.

aerosols, a provision of a biosafety cabinet is required. BSL-3 is used for the agents with a known risk of aerosol transmission and causing serious and potentially lethal infections. BSL-3 will have a biosafety cabinet and facilities such as safe handling, special clothing, minimum access, unidirectional airflow, etc. BSL-4 is a high-risk exotic agent which poses a risk of life-threatening diseases. There is no effective treatment against these disease-causing agents. Since these laboratories handle dangerous pathogens, they have highly advanced biosafety cabinets, positive pressure suits, filtered air facilities, airlock entry, and shower exits with special waste disposal mechanisms, etc. (Gupta et al. 2017). The assessment of biological risks related to mammalian cell cultures and the type of manipulation allows the determination of an adequate containment level to optimally protect human health and the environment. The list of general and more specific work practices and containment measures helps in the implementation of an appropriate containment level.

Along with the proper lab equipment usage and safety precautions, the labware and other consumables must also be sterilized or decomposed before sending it out to a healthy environment or before dumping in nature. This is the most important part of bioethics to look after the safety of the natural ecosystem. Further, the research study must go under the approval of the institutional-based bioethical panel to ensure the safety precautions taken up by the researcher or an organization. If the human intervention is included in the research study, then proper consent must be taken from the individual before starting the study. For example, isolation of a solid tumor from a patient and subsequently, using it for research purposes. This must be done *via* a proper channel of acquiring the consent from the patients and further giving them acknowledgment as and when possible of the findings.

Biosafety and bioethics are internationally recognized concepts. Biosafety refers to the maximal protection of the laboratory workers, public health, and the environment from the possible adverse effects associated with the use of organisms and microorganisms. Whereas, bioethics of mammalian cell culture enlists the limits of a modification allowed for a cell line beyond which it cannot be done to protect the environment around. It also provides the list of allowed modifications in the cell line and guidelines for the proper disposal of the sample before dumping it into the

natural environment. While working with the cell culture, the adverse effects related to the handling of cell cultures cannot be excluded, but a thorough risk assessment and the implementation of the appropriate containment level offer optimal protection for the laboratory worker and public health, and the environment. Further, because of the restricted survival capacity of the animal cell cultures in the *in vitro* environment, many cell lines are safe to use but may cause harm to humans, animals, or plants. The major hazard associated with the manipulation of cell cultures involves the fact that the manipulated cell may harbor adventitious agents, which are hard to detect and hence less controllable. These agents can survive in more hostile conditions and increase the risks for human health or the environment. Therefore, the assignment of the containment requirements cannot be generalized and should be performed case-by-case basis. If the containment measures are practiced properly, then the chances of adventitious contamination will be reduced, which will directly help in the minimization of the potential risks for the manipulator (Pauwels et al. 2007).

8 Significance of IPR on the Industrial and Academic Scale

IPR stands for intellectual property rights, which means it protects an individual's right against any novel findings, technology development, etc. There are three types of intellectual property rights, i.e., copyright, trademark, and patent. Copyright is the right that protects a tangible form of expression like a book, painting, etc. Copyright protects the mannerism in which the idea is expressed. On the other hand, the trademark refers to the protection of the logo or design that an individual or company uses. The distinguishable logo or design helps individuals to connect with the brand value and thereby ensures trust in the goods and services. Finally, a patent is used to prohibit the use, selling, by another party for a defined period, of the original production. IP rights are awarded to an individual or a company after a thorough examination of the invention and its viability. In the current era, getting intellectual property (IP) rights is to earn a profit on the innovative idea, encouragement of the ideas to secure them, business growth, and increasing the business opportunities. Brand recognition and financial income can only be earned on your inventions when you have rights on your research by trademark, copyright, or patent. For example, the patenting of the light bulb made the inventor recognize for their invention and also made huge money by selling the inventions to the customers. This is a classic example of the industrial aspect of the IPR. Whereas in the academic setting, the IPR protects the new findings by a researcher in a particular area and provides copyrights, patents to the researcher for their research. Without the presence of IPR, it would have been very tough for providing an individual their right to innovation, invention, and research. So, the understanding and acquiring of the IP rights either at an industrial scale or an academic scale is very important to give an individual their rights to innovation, invention, etc. (Morcos and Khneisser 2020).

9 Conclusions

The era of nanotechnology has just started to help in the advancement of current technology and novel invention. As the understanding of the biological interaction of the nanomaterials has increased, it has drastically increased the precision of the nanomaterial used in the biological field. This has also opened up a new area of research focusing on the possible toxicity from the nanomaterial and how to overcome that for an effective diagnosis and treatment. This has helped a lot in the drug delivery and fighting cancer, Alzheimer's disease, etc., effectively without excessive side effects due to the exposure of drugs to the normal tissues. Improvising the existing technology with the help of nanomaterials to have a higher sensitivity and more clearer and robust experimental setups. This is just the starting of the boon of nanotechnology to mankind. On the other side of it, the effect of the nanomaterials which are small to detect in the environment is still unknown and a future concern of the research. Further, the use of proper biosafety and following bioethics are important features of a good researcher. One should know the biosafety measures and the bioethics involved in the design research problem. Following it properly will help with the safety of the individual and of the cells we are working with. Also, the results will be trustworthy and reproducible if the proper safety and ethical measures are being taken care of by the researcher. Intellectual property rights (IPR) plays an important role in conserving your rights for the invention, findings, design, etc. At the industrial level, the company takes the right to earn profits on the research they have invested in. Whereas for the academic level, it gives the inventor its copyrights, patents, and identity of the novel research, inventions, or outcome. In the view of future, the more understanding of the nanomaterial will help in the advancement and ease of the human lifestyle. With these advancements, the stringency of the biosafety and bioethics of research will also help in the reproducible outcome and safety of the environment. The IPR aspects will help in keeping the copyrights, patents, etc., for the inventor and give them recognition for the research they have done.

10 Cross-References

- ▶ Emerging Drug Delivery Potential of Gold and Silver Nanoparticles to Lung and Breast Cancers
- ▶ Nanomaterials: Compatibility Towards Biological Interactions

Acknowledgments We also thank the funding support provided to us from the Science and Engineering Research Board (EMR/2017/002874) and the Department of Biotechnology (BT/PR26189/GET/119/226/2017) for the financial support.

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Emerging Drug Delivery Potential of Gold and Silver Nanoparticles to Lung and Breast Cancers

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_21-2

Abstract

The nanoparticles (NPs) of noble metals have emerged as biocompatible and site-specific drug delivery vehicles which reduce the undesired site effects and guard against accumulating toxicity. Herein, the drug delivery suitability of gold and silver nanoparticles is focused, owing to their enhanced use and multiple cell line compatibilities. The Au (atomic number 79) is placed exactly below Ag (atomic no. 47) in the 11th group of the periodic table, causing an easier Ag^+ generation than Au^+, having its valence electron much farther (in 6s subshell), exhibiting higher chemical stability. With such chemical interaction potentials, the Au is better suited as a capping and diagnostic agent, while the Ag aids in toxicity modulation within the tumor cells. The crux lies in transporting the maximum extent of delivered drugs to the needed location via guarding (stealth delivery) against a possible degradation by the physiological enzymes. Active and passive drug deliveries are the major mechanisms operational with NPs, with the former aimed at a direct approach to tumor cells, while the latter operates via drifting the physicochemical barriers in the tumor vicinity. The distinctive aspects conferred by the nanoscale attributes comprise enhanced surface area and facilitate high drug loading ability, ease of functionalization for targeted activities, and easy-to-follow robust bottom-up preparation methods. This chapter sheds light on the various Au and Ag NPs synthesis methods, their drug delivery attributes, a discussion of recent attempts toward lung and breast cancer treatment, and the cautionary aspects about the random toxicity of NPs.

Keywords

Drug delivery · Enhanced permeation and retention · Active and passive drug delivery · Ligand targeting · Au and Ag nanoparticles · Sustained release profile · Biodistribution · Pharmacokinetics

1 Introduction

Disease-free life is everyone's desire and irrespectively needed for wholesome societal progress. Increasing dependence on man-made resources and deviations from natural lifestyle propagation are the crucial factors responsible for enhancing the residence of chronic disorders. Nothing remains hidden in the light of recent turmoil generated by the residential effect of the SARS-CoV-2 pandemic, not sparing anyone across the globe. The intriguing factor has been the complication of issues that were once curable, owing to the manifestation of mutations and the ultimate development of resistant phenotypes. Although, plentiful curative options are available the essence of their specific suitability is the need of the hour. Furthermore, most of such options work via chemical modulation of the immune system through altered biochemical functioning, and exposing the body to unchecked exposure of these moieties results in unchecked stress build-up, which eventually manifests as troubling side effects. Numerous studies have noticed a side-effect manifestation exclusively due to unchecked dosage of the prescribed drug(s), whereby it is the desperate need of the hour to develop safer, sustainable, and structurally compatible delivery vehicles. For example, it is well known that drugs are chemical molecules that have an optimum pH and redox balance of functioning. Since the physiological pH varies in different compartments; it is much more likely that these molecules undergo a structural degradation due to unfavorable pH in the vicinity and by the time, a particular drug reaches the damaged tissue or its needed site of action, very little of it is left for its response delivery. Thereby, it is inevitable to have the vehicles which are made using low-energy methods (chemically more stable) and bind the drug being delivered via moderate noncovalent forces. At the same time, it is also essential that the release of drugs from the vehicle should be spontaneous based on a specific localized environment. If this process uses physiological energy, there are significant chances that the sufferer is exposed to undesired physical stress. Thereby, the drug and vehicle combination must not be chemically drastic and should be functional through a low activation energy provision. This brings us to the domain of biocompatible and multifunctional materials having high surface areas (SAs, suitable for high drug payloads) and are highly different from the classically energy responsive materials.

The nanomaterials are such divine assets for drug delivery that can be prepared using physical, chemical, and biological methods (unanimously aimed toward accomplishing chemical reduction) and have exceptional binding activities. These materials are the outcomes of quantum mechanics, wherein a discrete probability exists for every classically forbidden chemical event. A high surface area

(SA) confers these to have exponential binding abilities and thereby sue caution must be exercised to restrict the undesired chemical combination, which comprises another crucial factor in the physiological conditions due to accumulating oxidative stress. Such attributes ensure structural protection of bound drugs and ensure their sustainable release in a gradual manner contrary to an instantaneous regime which could aggravate the toxicity. The most significant advantage of such moieties as drug carriers is their ability to mediate a site-specific drug delivery, owing to which these can function with very low quantities of loaded drugs. Thereby, the chances of accidental drug spillage as well as elaborate patient sensitization are considerably reduced. The only factor which has posed caution to date after so much being well-known and understood is the excretion of nanoscale carriers. Several studies have raised concerns about such a possibility wherein the toxicity risk is manifolded by vulnerable cross-reactivity. Identifying the specific delivery site once the drug-loaded carrier is within the physiological boundaries, is a tedious task, for which active and passive delivery mechanisms are reported. Though the literature mentions these implicitly for tumor cells, these could be generalized for manifold cell-specific damages. The former approach targets the injured site through complementary binding with the cell-surface overexpressed protein while the latter exploits the troubled physiological vicinity of an injured cell and gains intracellular entry through accomplished physicochemical gradients. The details of these approaches are described in the text ahead, albeit the readers can consult the exhaustive literature for precise quantification.

This chapter, therefore, sheds light on the suitability of Au and Ag nanoparticles (NPs) for their drug delivery attributes with a discussion of the recent distinctive attempts and the emergent cautions that are needed to be exercised for a safer and least risk ensuring drug delivery.

2 Prominent Synthesis Methods of Gold and Silver Nanoparticles

2.1 Gold Nanoparticles

Nanoparticles of noble metals like silver, Ag, and gold, Au have been recognized for their very attractive size- and shape-dependent properties. As discussed above, the higher stability of Au makes it easy to functionalize for manifold analytical applications despite its working mechanism not being elucidated with a consensus. On the other hand, Ag has its valence electron in the 5s orbital and is smaller in size compared to Au. The spontaneous tendency of both Ag and Au to attain the nanoscale dimensions is optoelectronically related to losing their valence shell electron and forming the respective monovalent cations. The size differences between Au and Ag, with the former having its valence electron in farther 6s subshell, face a lower nuclear opposition than Ag, whereby Ag^+ is less stable and this distinction forms the basis of stronger Ag^+ toxicity. Perhaps that is the reason why nanoscale Ag is preferred for antimicrobial applications and is also included in

sun-screen lotions, soaps, and toothpaste with usage consent from the Food and Drug Administration (FDA).

2.2 Synthesis Methods of Gold Nanoparticles

The formation methods are distinguished based on their working principles of top-down and bottom-up approaches but the net aim of all methods is to accomplish the reduction of monovalent Au and Ag to the respective zero-valent states. The top-down approach works via cutting down on larger initial frameworks and generates a lot of waste. This approach is more used by physicists and enables a lower control on the size limit desired. In opposition to this, the bottom-up approach involves a combination of smaller molecular frameworks and is the method of choice for chemists. This approach generates much lower waste and enables a greater control toward controlling the size limit. With the availability of several robust chemical reducing agents, the chemical method of preparing the NPs is swiftly gathering momentum. The following paragraphs describe the various preparation methods of Au and Ag NPs. Owing to the formal ruling of not including more than 50 references, readers are requested to consult the 2014 review article by Herizchi and colleagues (for Au-NPs) and Iravani and associates (for Ag NPs), as these cover all the methods including their feasible extensions (Herizchi et al. 2014; Iravani et al. 2014). More specific references could be traced in these literature sources for implicit ideas of the studies. The major methods used to synthesize Au-NPs include chemical, thermal, electrochemical, sonochemical, and biological methods. The following paragraphs describe these methods in brief about their working principles.

2.3 Chemical Method

This chemical reduction-driven Au-NP *formation involves two steps*.

- Reduction of Au using suitable reducing agents such as amino-boranes, borohydrides, hydrazine, polyols, formaldehyde, hydroxylamine, sugars, oxalic acids/citric, H_2O_2, sulfites, CO, acetylene, H_2, and transition metal complexes.
- Stabilization of Au-NPs (maintaining a nanoscale size for a long) by capping agents such as sulfur ligands, trisodium citrate dihydrate, oxygen/phosphorus/nitrogen-containing ligands (heterocyclic compounds), polymers, dendrimers, leaf extracts, whole plant and fruit extracts and surfactants (ionic/nonionic).

2.4 Turkevich Method

- Based on reduction, Turkevich in 1951 developed a method using trisodium citrate for chemical reduction of Au precursor, such as $HAuCl_4$ to form

Au-NPs. In this method first, the aqueous solution of Au salt is prepared with optimized concentration. Thereafter, this solution is heated till boiling after which aqueous trisodium citrate is quickly added that resulted in a change from yellow to red color change (Fig. 1).

- This color change is an indication of formed Au-NPs, *vis-à-vis* altered optical, and physical properties, and a manifestation of quantum tunneling. This method is capable of forming 20 nm Au-NPs. The use of aqueous citrate solution gives the benefit of avoiding any one reducing or stabilizing agent because citrate ions work as reducing as well as a stabilizing agent (Turkevich et al. 1951). The method can provide 150 nm Au-NPs by varying the mutual stoichiometries of Au precursor and reducing agent (citrate solutions). Using this method, several shapes, including rods, stars, ellipsoidal and spherical, could be formed. The method enjoys wide acceptance as it is highly reproducible and takes less time to form the monodispersed and highly stable Au-NPs.
- For instance, a low concentration of aqueous citrate results in aggregation and hinders the formation of monodispersed Au-NPs whereas a high citrate concentration provides highly stable and monodispersed NPs (Frens 1973). Besides the citrate concentration, other notable parameters affecting the process include pH, temperature, and time duration (mentioned in terms of aging) changes which could result in varying dimensions of as-formed Au-NPs. Variation in sizes and shapes is mentioned in Table 1 where high pH favors spheroid particles while at lower pH the polyhedral morphology is obtained. At low pH, the diffusion-

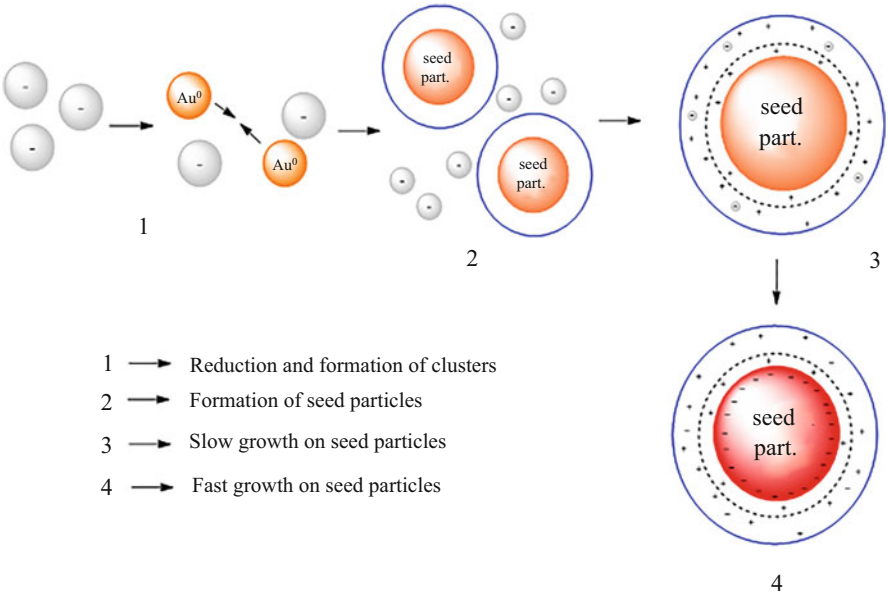

Fig. 1 General growth mechanism of Au-NPs using the Turkevich method

mediated aggregation decreases the surface energy to form polyhedral morphologies.

2.5 Brust–Schiffrin Thermal Reduction Method

- This method uses thermal energy for the formation of stable Au-NPs (thermally and air) with desired size and low polydispersity. In this approach, the transfer of Au salt ($HAuCl_4$) to an organic phase like toluene forms the aqueous phase by tetrabutylammonium bromide (TOAB) which is used as a phase-transfer agent (Fig. 2). Thereafter, $NaBH_4$ is added in presence of dodecanethiol that, resulting in deep brown color from the previous orange, confirming the Au-NPs formation (Burst et al. 1994).

2.6 Digestive Ripening Method

- This is a convenient technique for the preparation of monodispersed Au-NPs with the excess ligands or peptic ripening agents where a colloidal suspension containing Au salt is heated at about 138 °C for 2 to 3 min. After this, it is heated again at 110 °C for 4 to 5 h in presence of a capping agent, which is alkanethiol. This is the thermal method where temperature controls the size and shape of the Au-NPs.

Table 1 pH-dependent shape and size diversity of Au-NPs

pH	Size	Shape
2	(20–30) nm	Mirror-like deposit
3.5	(30–60) nm	Dispersed
4	100 nm	Polyhedrons
4.5	100 nm	Crystal faces
5		
5.5	100 nm	Oblate
6	50 nm	
6.5	50 nm	Spherical

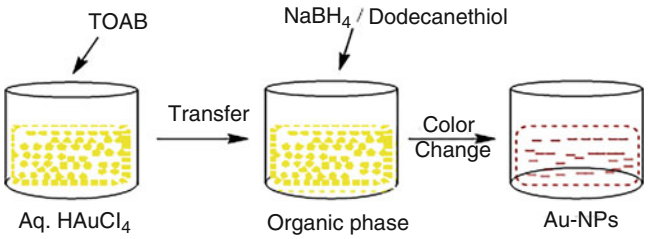

Fig. 2 The Brust–Schiffrin method for preparation of Au-NPs

2.7 Electrochemical Method

- For the size-selective Au-NPs, the electrochemical approach was first introduced in 1994 by Reetz and Helbig. In this method, the tetra alkyl ammonium salts are used for stabilizing Au-NPs within a nonaqueous medium. This method uses cathode and anode as electrode cells; where Au-NPs are deposited on the surface of the cathode which is made up of multiwalled carbon nanotubes having a glassy texture.
- Apart from this, the electrochemical method is robust with an inferior processing temperature, better quality, and less cost, enabling better quality control. Figure 3 depicts the experimental setup for the formation of Au-NPs using the electrochemical method.

2.8 Seeding Growth Method

- This method is specifically suited for the preparation of 5- to 40-nm diameter Au-NPs in a monodispersed state. For controlling the sizes, the seed to metal salt stoichiometry is optimized where trisodium citrate and sodium borohydride ($NaBH_4$) are used as sources of hydroxide ions and reduction, respectively (Siti et al. 2013).
- This is a *bottom-up method and forms the nanomaterial* in a layer-by-layer (epitaxial) manner. Figure 4 depicts a general experimental setup and mechanism for seeding growth method-driven Au-NPs preparation with oleylamine as a reducing and stabilizing agent.

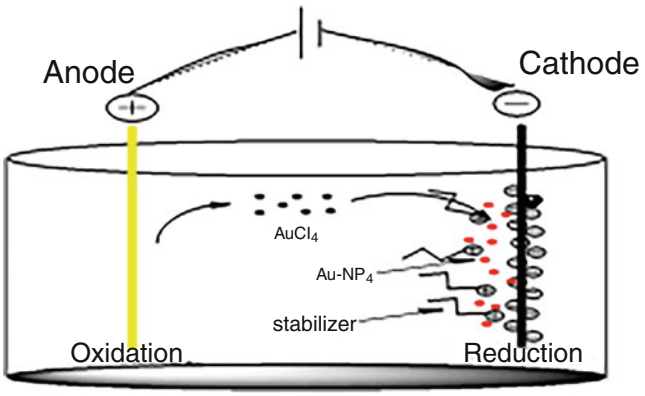

Fig. 3 The set-up for electrochemically prepared Au-NPs formation. The charge-driven reduction attained via opposing proximities is the principle of this top-down approach. Variation in sizes could be obtained via changing electric current input

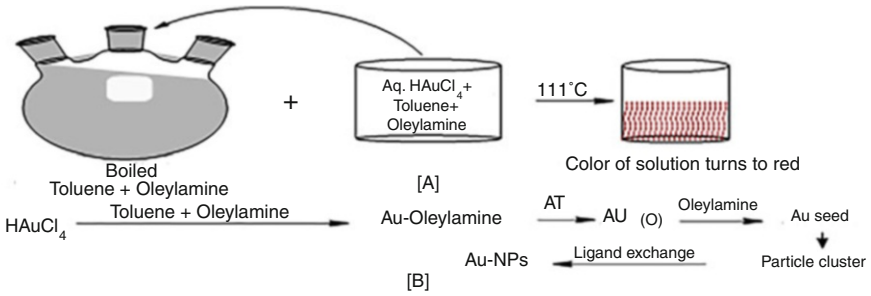

Fig. 4 Preparation of Au-NPs (Seeding growth method), [A] experimental setup [B] mechanism

2.9 Ionic Liquids-Mediated Gold Nanoparticle Formation

- Synthesis of Au-NPs using ionic liquids (ILs) has been found as an alternative to other methods for the same. ILs provide a significant medium for the preparation of Au-NPs with stabilization because of their thermal stability, nonvolatility, ease of miscibility with cosolvents, and low melting point (Richter et al. 2013). These ILs also function as a capping agents, templates as well as precursors of the materials. In this context, the functionalized ILs are being used for the one-phase synthesis of Au-NPs with enhanced and longer stability.
- The number and position of functional groups associated with ILs affect the formed Au-NPs in terms of diameter and stability. Alcoholic groups of ILs act as reducing agents as well as a stabilizer and because of this, they are considered for making the NPs formation robust. In this regard, the use of quaternary ammonium ILs are considered for preparing stable Au-NPs where the *chelating ability of ILs* performs a role of reducing and stabilizing agents. Figure 5 portrays a general sketch of the prepared Au-NPs using ILs.
- The imidazolium cation-based ILs have been used for making Au-NPs which have been found effective to screen an aggregation *vis-à-vis* color changes in an aqueous medium as a visual device for anions.

2.10 Vacuum Sputtering Method

- This method uses a vacuum chamber having two electrodes in which an inert gas is passed and ionized in the presence of electricity. This generates a potential difference between the two electrodes. Argon (an inert gas) plasma is bombarded to the cathode (metal to be converted into NPs) resulting in punched atomic clusters from the cathodic target area getting deposited in the solution.
- This method is highly robust and forms pure Au-NPs because other than Au, no chemicals are precipitated (Fig. 6). In this method, the ILs are used for the stabilization of Au-NPs due to their low vapor pressure, an essential requirement for the preparation of solutions by cathode sputtering.

Fig. 5 Stability of Au-NPs using functionalized ILs. The versatility of ligand choice in ILs can couple a range of reducing agents to the being prepared Au-NPs surface

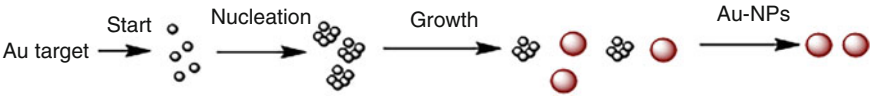

Fig. 6 Vacuum sputtering method for preparation of Au-NPs

Scheme 1 Formation of hydroxyl free radicals by the dissociation of water

$$H_2O \longrightarrow {}^{*}OH + {}^{*}H$$

$$RH + {}^{*}OH\ ({}^{*}H) \longrightarrow {}^{*}R + H_2O\ (H_2)$$

RH = pyrolysis raical and unstable product

$$Au(III) + \text{reducing agent}\ ({}^{*}H, {}^{*}R) \quad Au(0)$$

- Upon the needed extent of capturing medium and under optimum deposition conditions, an optimum yield of ILs and vegetable oils is obtained.

2.11 Sonochemical Method

- It is an advanced technique for the preparation of metallic NPs with a *smaller size and a rapid reaction rate*. The wide size distribution is the limitation of this method but using surfactants and alcohols, the desired diameter and geometry could be obtained. In a sonochemical method for preparing Au-NPs through reduction of Au (III) using organic additive, the following steps are considered (Scheme 1, below).

2.12 Reverse Micelles Method

- This method uses *thermodynamically stable microemulsions* formed by having two immiscible liquids with a suitable surfactant for forming Au-NPs with controlled diameter and shape. The emulsion results in the formation of micelles that are highly effective for NPs stabilization via larger SA and long-ranged

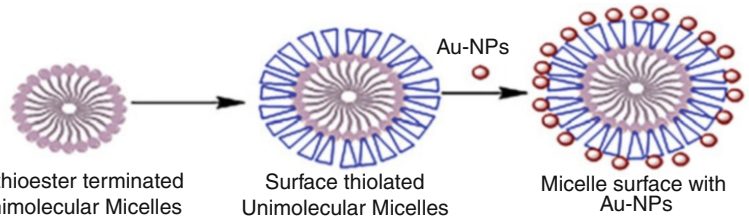

Fig. 7 Preparation of Au-NPs using the reverse micelles method

$$CH_3COCH_3 + (CH_3)_2CHOH = 2(CH_3)_2(OH)C$$

Scheme 2 Formation of ketyl radicals via excitation of acetone with ultraviolet radiation

molecular interactions. Figure 7 depicts the micellar-stabilized Au-NPs attained via surface functionalization.

2.13 UV Light Irradiation Method

- The ultraviolet irradiated synthesis of Au-NPs involves a stabilization of aqueous $AuCl_4^-$ using an organic solvent such as 2-propanol, or acetone with polymeric stabilizers (Scheme 2). The organic solvents are excited under UV light, which results in the metallic reduction and the subsequent NPs formation (Henglein 1999).

2.14 Biological Method

- This method of NPs preparation either *uses leaf/fruit extracts or microbial enzymes* for the formation of NPs. Extracts from different plant sources are comprised of polyphenols, alkaloids, and other phytochemicals which are green and renewable. These materials provide hydrophilic and hydrophobic force gradients in their liquid state and induce *moderate capping with no harsh structure-breaking activities*.
- Similarly, enzymes from microbes are highly suited reducing agents for the metals from their mono/di valent state in their precursors to a zero-valent state. Microbes are capable of *extracellular as well as the intracellular* formation of NPs and can even use deleterious waste streams as feed inlets (for culturing) to form metal NPs.
- A simple flow diagram for the preparation of metal NPs using the biological method is depicted in Fig. 8. Though plant extract(s) and microbial enzymes are renewable resources and generate no environmental hazard during the NPs formation, their use as capping agents is not as efficient as those of chemical

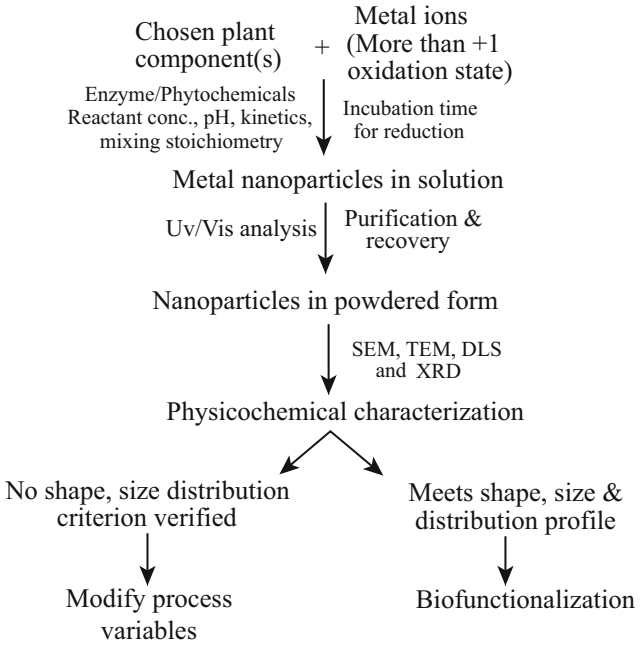

Fig. 8 Plant sources-mediated preparation of Au-NPs, the renewable nature of microbial enzymes and plant extracts (leaf, fruit, and even whole plant) makes this method less dependent on external energy

reducing agents. This is why the NPs prepared using the biological method are often larger or undergo *time-dependent aggregation/coalescence*, making them unsuitable for specific, size-dependent applications.

- For the preparation of intracellular and extracellular Au-NPs, different microorganisms are considered because bacteria develop a negative charge on their cell wall which can easily intercept Au(III) ions having a positive charge. In the case of intracellular synthesis, initially, the Au(III) ions are transported into the cell wall then the enzyme and other proteins carry them for the synthesis of NPs. While in the case of the extracellular synthesis process, the Au(III) ions are captured by the cell membrane enzyme those are reductase enzyme, and then these enzymes reduce Au(III) and synthesize Au-NPs outside the cell.
- The electrons utilized in Au(III) to Au(0) reduction are provided by NADPH through electron transfer chain enabled NAD^+ formation. To prevent agglomeration, the microbial cell produces certain enzymes, proteins, and organic material which perform the role of a capping agent to keep the Au-NPs monodispersed.
- The reduction by microorganisms is efficient for narrow size distributions and monodispersity of Au-NPs wherein the geometry and diameter can be controlled by changing the microbial culture constituents.
- Similarly, fungi are another natural source for preparing Au-NPs which conceal metabolites, and extracellular enzymes such as acetyl xylem esterase,

3-glucanase, hemicellulose, and cell wall lytic enzyme. Fungal-mediated intracellular preparation of Au-NPs involves bio-reduction of Au(III) by reductase. Apart from this, the lactase secreted by fungi is also suited for the extracellular biosynthesis of Au-NPs.

2.15 Biomolecule-Driven Gold Nanoparticle Formation

- For speeding up biological processes, the biomolecules are synthesized by living organisms such as macromolecules, nucleic acids, amino acids, lipids, and carbohydrates. For example, chitosan which is a polysaccharide has been used as a reducing and stabilizing agent for the preparation of Au-NPs.
- Another polysaccharide such as starch is also used for the preparation of Au-NPs in an alkaline environment, where carboxyl and the hydroxide groups perform the reduction of Au^{3+} to Au-NPs. Similarly, the proteins are also used for the preparation of Au-NPs.
- The advantage of using proteins and polysaccharides for Au-NPs preparation *is their larger SA-driven distributed interactions* which prevent the aggregation in NPs. The segregated domains in polysaccharides and the multifunctional substituents of protein amino acids not only functions capably as reducing agent but also guard (*through its van der Waal forces, hydrogen bonding, and London Dispersive Forces*) against random aggregation.
- Many times, applications of biological or sensing domains require the functionalization of probes, which can be easily accomplished using biomolecules. Polymeric networks like those of glutaraldehyde, pullulan, xylose, and other polysaccharides are well known for their dispersion-enhancing philophobic force gradients.

2.16 Synthesis Methods of Silver Nanoparticles

- Similar to Au, several nontoxic and green chemistry-based methods have been optimized for preparing Ag-NPs through their specifically tuned reducing ability. Recently many green methods have been developed that involve polysaccharides, polyoxometalates, biological, tollens, and irradiation techniques.
- An overview of various methods for Ag-NPs formation with specific reducing and stabilizing agents along with implicit size limits is described in Table 2. These methods exhibit manifold advantages over conventional approaches in their robust activities, and the use of eco-friendly solvents and chemicals for reduction and stabilization.
- Some frequently practiced processes for the preparation of Ag-NPs are chemical and physicochemical reductions, electrochemical techniques, and radiolysis. While aggregation stability remains the most important criterion for choosing a particular synthesis method, attempts toward improved countering of particle sizes, stability, and aggregation problems.

Table 2 An overview of salient prospects of physical, chemical, and photochemical methods for synthesizing Ag NPs (Iravani et al. 2014)

Source	Method	Reducing agent	Stabilizing agent	Size (nm)
$AgNO_3$	CM	DMF		<25
$AgNO_3$	CM	$NaBH_4$	Surfactin	3–28
$AgNO_3$	CM	Tri sodium citrate	Trisodium citrate	<50
$AgNO_3$	CM	Tri sodium citrate	Trisodium citrate	30–60
$AgNO_3$	CM	Ascorbic acid		200–650
$AgNO_3$	CM	$NaBH_4$	DDA	7
$AgNO_3$	CM	Parafin	Oleylamine	10–14
$AgNO_3$	CM (Thermal)	Dextrose	PVP	22–25
$AgNO_3$	CM (Thermal)	Hydrazine		2–10
$AgNO_3$	CM (Oxidation of glucose)	Glucose	Gluconic acid	40–80
$AgNO_3$	CM (polyol process)	Ethylene glycol	PVP	5–25
$AgNO_3$	CM (polyol process)	Ethylene glycol	PVP	50–115
$AgNO_3$	Electrochemical (polyol process)	Electrolysis cathode, Titanium anode. Pt	PVP	11
$AgNO_3$	CM (Tollen)	m-Hydroxy benzaldehyde	SDS	15–260
Ag wires	PM	Electrical arc discharge, Water		10
$AgNO_3$	PM	Electric arc discharge	Sodium citrate	14–27
$AgNO_3$	CM (microemulsion)	Hydrazine hydrate	AOT	<2
$AgNO_3$	PR (microwave radiation)	Ethylene glycol		<2
$AgClO_4$	PR (pulse radiolysis)	Ethylene glycol	PVP	5–10
$AgNO_3$	PR (Photo reduction)	UV light		4–10
Ag_2SO_4	PR (X-ray radiation)	X-ray		2–8

CM chemical method, *PM* physical method, *PR* photochemical reduction

2.17 Physical Methods

- These methods generally follow the principles of top-down methodology, using a bulkier template and gradually modifying it to reduced dimensions. A major challenge with these approaches is their dependence on external energy which is why the stability of NPs using these approaches is not too high. Analogous to this, these methods generate a lot of waste materials and are therefore a threat to the engaged researchers and persons in the vicinity.
- Generally, the evaporation-condensation and laser irradiation are included in the category of physical approaches where null solvent contamination and uniformity of Ag-NPs distribution are the benefits. Some concerns of this method include the use of tube furnaces needing large space, energy, and longer processing time for achieving thermal stability.

- Instead of the furnace, the small ceramic heater is used for the same purpose which evaporates the source and forms small-sized Ag-NPs with large concentrations. Here, the temperature of the heater surface is time-independent so the formed NPs are found to be very stable.
- The laser processing technique is useful to prepare *pure and uncontaminated* Ag colloids without the use of chemical reagents. This technique is different from other physical methods where the irradiation of the Ag sheet is initially engrossed in an aqueous surfactant solution using a pulsed laser (Fig. 9).
- The typical intensities and working modes of laser ablation affect the size, shape, and growth of Ag-NPs. In this method, a spherical morphology of Ag-NPs drives the mechanism of **vapor-liquid-crystal condensation**. Laser intensity need not be too high although it is the energy source for breaking the larger basal platform into small structures (the top-down principle). The *duration of laser* exposure and the *nature of the salt precursor* is the major criteria for obtaining the NPs of the desired configuration, using this method.

2.18 Chemical Reduction Method

- Preparation of Ag-NPs through chemical reduction is most popular and preferred due to ease of synthesis in solutions with variable sizes and shapes. For instance, in the polyol process, the development of monodisperse Ag-nanocubes via reduction of silver nitrate with ethylene glycol (EG) in the presence of a polymer like polyvinylpyrrolidone is done (Iravani et al. 2014).
- Here EG acts as a solvent and reducing agent, both. Typical $AgNO_3$ concentration and molar ratio with the nature of polymer are size deciding factors of the nanomaterials desired. It is a most common technique for the preparation of Ag-NPs where for reduction purposes organic and inorganic substances are used such as ascorbic acid, sodium citrate, $NaBH_4$, H_2, Tollens reagent, polyol process, EG, and DMF (N, N-dimethylformamide).
- Typically, a reduction of Ag^+ forms metallic Ag due to agglomeration, resulting in the formation of oligomeric clusters of Ag which provide Ag-NPs (Fig. 10). The

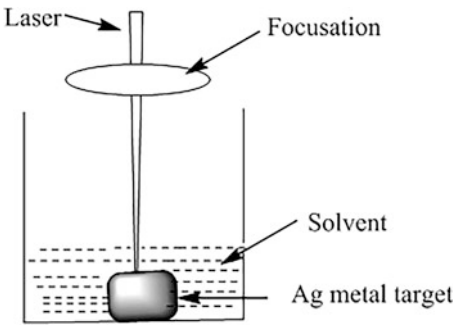

Fig. 9 Laser ablation technique for the preparation of Ag-NPs

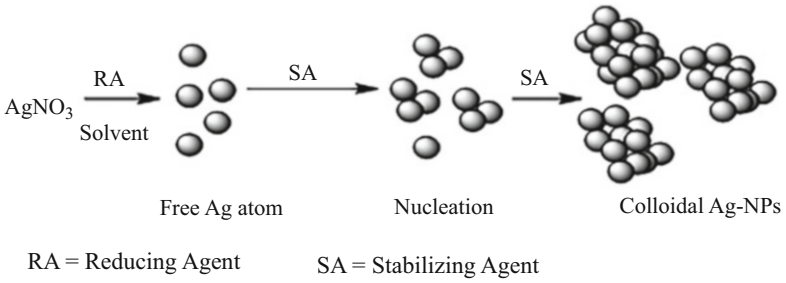

Fig. 10 Chemical reduction approach for preparation of Ag NPs

stabilization and self-agglomeration of Ag-NPs are avoided by surfactants containing amines, thiols, acids, and alcohols as functional groups which interact with NPs surfaces. Not only surfactants but also poly (vinyl alcohol), EG, vinylpyrrolidone, methacrylic acid, and methylmethacrylate are also used for capping activities.

- In general, Ag source material, and reducing and stabilizing agents are the three major requirements for preparing Ag-NPs using the chemical reduction method. The diameter and shape of being prepared NPs depend on nucleation followed by stacking of the Ag nuclei where uniform size distribution is obtained through optimum selection of reducing and stabilizing agents along with controlling the reaction time.

2.19 Microemulsion Techniques

- Of the foremost techniques to prepare homogenously dispersed Ag NPs, the green route of microemulsions uses Ag metal precursor (a suitable salt) as aqueous and a compatible reducing agent, as organic phase, respectively (Fig. 11).
- The immiscible phases (Ag metal precursor and reducing agent) are made to interact using an emulsifier such as quaternary alkylammonium compound which affects the reduction rate. The formed clusters are stabilized in the aqueous phase and transferred to the organic phase using the emulsifier.

2.20 UV-Initiated Photoreduction

- In this method, the reduction of Ag^+ is done in the presence of polyvinylpyrrolidone (PVP), citrate, poly acrylic acid, and collagen under the simultaneous influence of UV radiations. This method enables significant control of the size and dispersion patterns of the synthesized Ag NPs.
- Exposure to UV radiations and the kind of precursor being used are the critical factors that can regulate the characteristic shape and size of the nanomaterial(s).

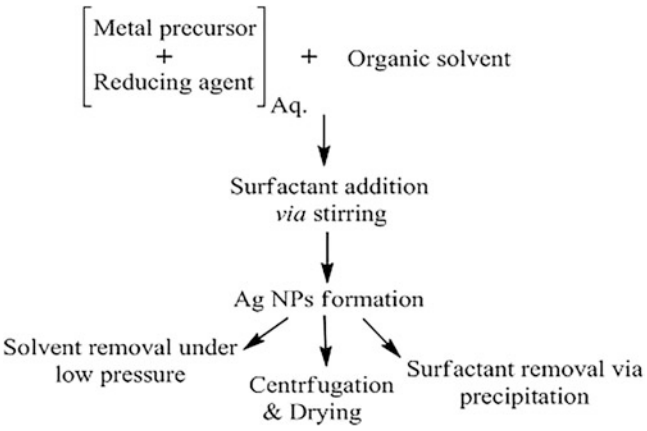

Fig. 11 Microemulsion technique for preparation of Ag-NPs

Using this method, it is possible to reduce the different kinds of salt precursors to a monovalent state, including those of natural minerals and synthetic complexes. The mechanism relies on the distinct response of constituent moieties toward the UV light. One study reported the formation of wire and sphere morphologies from the same precursor, synthesized using the same technique where polyvinyl alcohol (PVA) acted as a stabilizing agent.

2.21 Electrochemical Reduction Method

- The electrochemical method which involves the electrolysis process uses a charge-controlled electrolytic reduction of salt precursors to their nanoscale form. For example, the polymer-coated spheroid Ag NPs with 3 to 20 nm are prepared using this method at the liquid/liquid interface. Similarly, zeolite-modified electrodes are being used for making (1–18) nm Ag NPs with a monodispersed distribution regime (Zhang et al. 2002).
- Suitable polymers (ensuring wider ranged binding forces with maximum VWF), such as PVP and others, can act as the stabilizer to protect the NPs from agglomeration which reduces the Ag deposition rate alongside promoting NP formation.

2.22 Microwave-Assisted Synthesis

- It is an advanced but green approach to preparing Ag-NPs, specifically with smaller, narrower size distributions and a higher degree of crystallization (Fig. 12). This technique consumes *less reaction time and energy*, giving higher

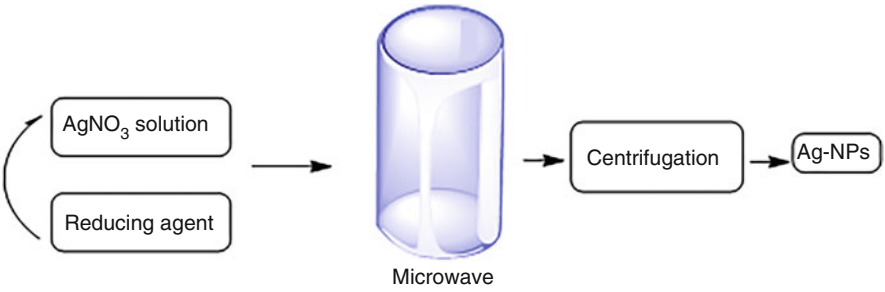

Fig. 12 Microwave method for the synthesis of silver nanoparticles

yields and minimizes the agglomeration, is known as a green synthesis of Ag-NPs.

- The sodium carboxymethyl cellulose is used as a reducing as well as stabilizing agent where the cellulose and $AgNO_3$ concentrations are the regulating factors for the size and stability control of NPs. The *microwaves are penetrating energy sources* and work via dissociating the salt precursor. Using this method, Ag-NPs have been prepared with Pt seeds, ethylene glycol, PVP, and starch in smaller sizes.
- The method has been successful for the large-scale requirement of NPs and provides an adequate amount of desired sized Ag NPs using sodium citrate and $AgNO_3$ in an aqueous state through slow but gradual microwave irradiation. The process uses formaldehyde (a reducing agent) where the state of Ag^+ directs the size and distribution. Using amino acids and starch as reducing and stabilizing agents fetches monodispersed Ag-NPs.

2.23 Polymers and Polysaccharides-Based Synthesis

- This method is eco-friendly, using water as a solvent with polysaccharides as capping or reducing agents. The polysaccharides generally interact with Ag-NPs through weaker binding forces but at higher temperatures this interaction could be reversed which allows separation of NPs. Here the Ag^+ reduction happens in an environment of nanoscale starch templates where the philophobic force gradients and weaker interactions (sensitive to functional SA) guard against a vulnerable random aggregation.
- Figure 13 presents a general scheme of polysaccharide capped formation of Ag-NPs, where the NO_3^- of $AgNO_3$ precursor abstracts the H^+ of –OH groups in the β-glucose structure. As a result, the formation of NPs has <7 pH as its ambient environment with enhanced stability of medium driven via anionic conjugate formation of β-glucose.

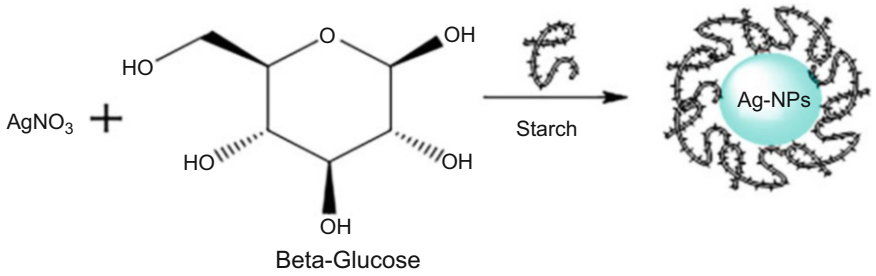

Fig. 13 Starch encapsulated formation of monodispersed silver nanoparticles

2.24 Tollens Method

- This is a green method to synthesize controlled size Ag NPs via chemical reduction of Tollens reagent $Ag(NH_3)_2OH$ using aldehyde (Yin et al. 2002). The method could be modified using polysaccharides instead of aldehyde which reduces Ag^+ with NH_3, generating *50 to 200 nm Ag-NPs and 20 to 50 nm Ag hydrosols*. For instance, the low concentration of NH_3 (5 mM) and glucose produce 57 nm Ag-NPs whereas a high concentration increases the size and PDI.
- The reduction is significantly affected via medium choice where the hydrophilic and hydrophobic activities differently engage NH_3^+ and OH^-. Another caution pertains to the necessity of making Tollen's reagent freshly each time the reaction has to be conducted, which is *due to the short half-life* of this reagent in its native state.

2.25 Biological Sources-Mediated Precursor Reduction

- The chemical methods for Ag NPs preparation are not eco-friendly and expensive, therefore, the manifold biological sources ensuring an environment-friendly reduction of salt precursors have been developed. Such methods do not use any toxic chemicals and rely on plant extracts and microbes as reducing agents, just like that of Au-NPs. Readers are requested to have a look at the more specific literature sources to know the details about these methods with the varied controls and differential synthesis conditions.
- Biological agents (microbial enzymes and plant extracts) enable the formation of highly stable and well-characterized Ag-NPs via optimal growth and culture conditions. By altering the physiological parameters such as concentration of substrate, light, pH, electron donor, temperature, and buffer strength, the sizes and shapes could be controlled.
- This is a green method and uses *biocompatible molecules* (plant extracts and microorganisms, enzymes) as reducing and stabilizing agents. A significant concern is the vulnerability of as-formed NPs toward getting agglomerated

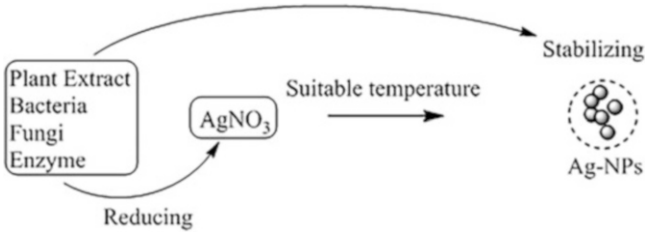

Fig. 14 Green synthesis of Ag-NPs using renewable energy routes

since the capping of plant extracts and microbial enzymes either develop self-assembly (due to manifold –OH groups) or the functionally integrated *nanoionic hydration spheres* (NIHS). Figure 14 outlines a general scheme for the formation of Ag NPs using this method.

3 Gold and Silver Nanoparticles as Drug Carriers for Lung and Breast Cancer Treatment

- Though interest in nanoparticle-mediated drug delivery continues to fascinate scientists and researchers for a long, there has been extensive use of Au-NPs for biosensing, drug delivery, and Ag NPs for targeted toxicity induction. Although the recent emergence of functionalized graphene derivatives, silica and manganese conjugated oxide NPs have gained interest for diagnostic purposes drug delivery studies and investigations have been majorly attempted using Au and Ag NPs.
- Reasons for such significant interest in Au and Ag NPs are linked with their electronic configurations, with Ag coming earlier than Au but the placement in the same group of periodic table is the reason for most of the chemical properties being similar to each other. With a farther placement of the outermost electron for Au than Ag, Au is comparatively more stable than Ag. Perhaps this seems the reason for the auspicious and increasing application of Au-NPs in drug delivery as an inherent low reactivity makes Au-NPs easy to functionalize and be conjugated with drug delivering cargos. The mechanism of action is albeit, more well understood for Ag NPs, which exert their antimicrobial response through Ag^{+} formation. For Au, there is an additional benefit of being used as a surface capped layer, since the Au periphery would minimize the random and nonspecific interactions and also ensure a prevalence of nanoscale texture for the underlying assembly.
- Figure 15 depicts the major Au and Ag NPs distinguishing abilities for drug carriers. Methods as diverse as using vegetable and fruit peels along with their extracts are reported for mono to zero-valent reduction of the metal salt precursors. The approaches using physical stimulus are described by the top-down principle, wherein a lot of waste material is generated besides the limitation of not

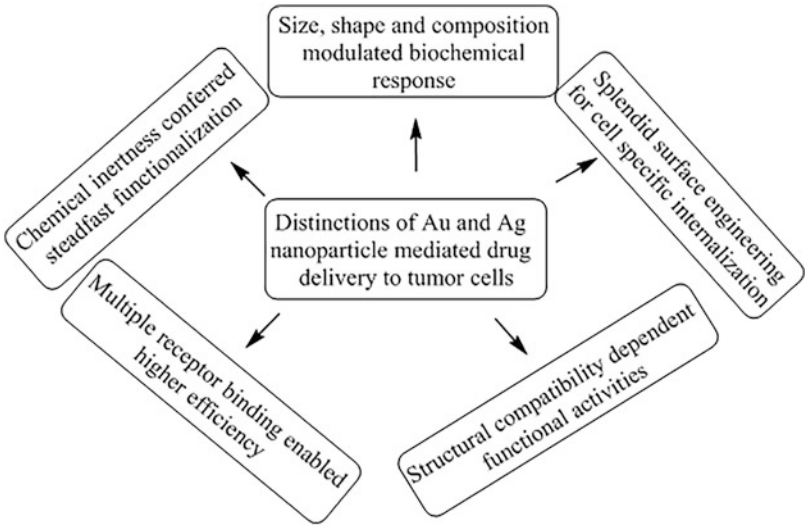

Fig. 15 The characteristic benefits of Au and Ag nanoparticles for their drug delivery applications. The major distinctions include shape and size modulated performance, robust surface functionalization mechanisms, and simplistic preparation methods

stopping the process in between. Contrary to this, the mechanism followed by chemical reduction relies on a bottom-up methodology with little wastage and the advantage of terminating the reaction as per the specific requirement.

- It must be noted here that the availability of manifold handy reducing agents makes the chemical methods more suitable and versatile. For Au-NPs, as also stated above, the *Turkevich method* has been demonstrated with immense reproducibility with the varying precursor to reducing agent stoichiometries being capable to produce variations in the NPs sizes. The advantage of the chemical method lies in the substantially higher stability of prepared NPs compared to those of physical and biological, both of which exhibit aggregation concerning time and usage multiplicities. The methods involving using leaf and fruit extract as reducing agents for chemical precursor reduction are suitable at the laboratory scale and are relatively cumbersome to meet the pilot scale requirement.

4 Mechanisms of Nanoparticle-Mediated Drug Delivery to Cancer Cells

- Delivering drugs through NPs to cancer cells is facilitated via active and passive targeting. The challenge in the overall process pertains to the discrete identification of tumor cells which is further compounded by the concomitant risk of arresting the metastasis. The intent of drug consumption pertains to induce a characteristic change in the biological response subject to its known chemical

structure. Typical hallmarks encountered by a trafficked drug in its passage to tumor cells comprise the troubled extracellular environment, the overexpressed cell surface receptor proteins, unusually faster-growing pace, and loss of cell-cell communication. Apart from this, the ability to form new blood vessels for the circulation of nutrients, named angiogenesis is a significant feature of tumor cells for their distinction from normal cells. Since the challenge lies in adequate identification of tumor cells, the mechanisms are therefore recognized through their site-specific activities and are hence termed as active and passive targeting. Another aspect worth noting is that there is an exclusive mention of these mechanisms concerning tumor cells, primarily because interventions like NPs as drug delivery carriers are screened only in extreme cases or in terminal situations. In general, the fundamentals of delivery mechanisms are feasible in nontumor cells also. As the present chapter is specifically focused on the anti-cancer efficacy of Au and Ag NPs, so the major aspects of nanoparticle therapeutics via active and passive targeting are mentioned in subsequent paragraphs. Readers are suggested to refer 2014 contributions of Bertrand and accomplices (from Advanced Drug Delivery Reviews) and 2018 of ours (focused on Au and Ag NPs treated lung and breast cancers) for all aspects in the subsequent discussion on active and passive drug delivery as formal bindings necessitate not including more than 50 references (Bertrand et al. 2014; Malik and Mukherje 2018).

4.1 Active Targeting

- Active targeting is attributed to the identification of a nanoparticle surface ligand by the tumor cell-specific over-expressed surface receptor(s). This approach involves considerable risk as any random misfired delivery is likely to harm the normal cells and the damage would be further manifold due to functional cell-cell signaling in normal cells. The enhanced complexity of this delivery mechanism necessitates that the ligand-specific antigen (or receptor) is not merely present on the tumor cells but also remains accessible to the administered NPs. Table 3 provides some fundamental information about the various ligands used for targeted drug delivery using NPs along with their specific limitations, unique aspects, and the current progress of the work.
- Another essential factor regulating the success of active targeting is the chance of inadequately targeted receptor localization and expression which could weaken the ligand binding. Exclusive ligand moieties used in the active targeting approach include antibodies, peptides, nucleic acids, sugars, and several erstwhile small molecules such as vitamins. Likewise, the target moieties can be proteins, sugars, or lipids prevailing on the tumor cell surface.
- The outcome of active therapy depends on multiple factors, the major among which is the preciseness with which ligand conjugated drug-loaded NP complexes are targeted to the surface receptors of tumor cells. Hence, the specificity with which the drug-loaded NPs are targeted to the cancer cells holds prominence

Table 3 The various ligands used for targeted drug delivery via nanoparticle involvement, small molecules represent the ones with emerging significance. Aptamers also find some specific suitability and have superseded the aspirations of monoclonal antibodies

Ligand configuration	Fundamental essence	Unique feature	Work in progress and concurrent restrictions
Monoclonal antibodies (MAbs)	Engineered MAbs capable of escaping immune detection have been used to develop targeted NPs,	Ability to target and interfere multiple cellular processes, Rituximab and Trastuzumab enable enhanced uptake on being conjugated to PLA NPs	Chimeric and humanized derivatives are being developed for modulating immunogenicity, large size, extensive optimization, cumbersome nanoparticle scale up and immunogenic traits could result in rapid clearance of NPs, shifted the interest to less immunogenic and lower sized Ab fragments
Aptamers	Small, single stranded DNA/RNA oligonucleotides sequences less than 15 kDa in size, have less immunogenicity, better stability and an improved biodistribution	Exhibit folding ability via intramolecular interactions to unique three dimensional conformations and topologies needed for efficient target affinity	Select aptamers with a specific target binding are being developed via Systemic Evolution of Ligands by Exponential Enrichment (SELEX), Customized Customized-controlled release docetaxel-loaded NPs proximal for prostate specific membrane antigen (PSMA) have been reported, PSMA over-expresses in prostate and on neovasculature of many solid tumors, a clinically approved targeted polymeric nanoformulation (BIND-014) has been developed
Proteins and Peptide-based targeting molecules	Emerging attractive targeting molecules having a small size, high stability, and low immunogenicity compared to many proteins	Can bind to multiple molecular targets with high affinity and specificity, easy to manufacture by conjugation to polymers, lipids, and many NPs surfaces	An iron transporting protein, transferrin binds specifically to transferrin receptors on the cell surface for transporting NPs to different cells, targeting efficacy is

(continued)

Table 3 (continued)

Ligand configuration	Fundamental essence	Unique feature	Work in progress and concurrent restrictions
			impaired by immunogenicity and early clearance
Small molecules	Low molecular weight (<500 Da) organic molecules	Small size, high stability, chemical management, and low production cost enable facile coupling methods for conjugation, fewer in vivo immunogenic effects, reproducible and economic manufacturing	Folic acid is the most studied ligand for drug delivery, targets overexpressed folate in multiple tumors, the pseudomimetic dipeptide 2-{{(5-amino-1-carboxypentyl) carbonyl]amino} pentanedioic acid has been used to develop (-)-epigallocatechin 3-gallate-loaded PSMA targeted NPs, carbohydrates, sugar-based compounds having low molecular weight, biocompatibility and ease of production, are being explored as ligands for cell-specific drug delivery

so that the effect of administered drugs remains localized to cancer cells. A conditioning element of active targeting mandates the delivery of drug-loaded NPs enriched cargos in the vicinity of targeted tumor cell surface receptor proteins. This facilitates the required recognition for optimum interaction. This attunes the blood supply to the tumor cells on account of which, the affinity with which drug conjugated NPS targets the tumor cell can never exceed the physiological detection and elimination provisions. Overall, this facilitates a longer persistence of drug-loaded NPs with improved pharmacokinetics and sustained response.

- The above constraints necessitate configuring NP architecture equivalent to escape immune system detection with a long-lasting blood circulation time. These factors illustrate an ultimate dependence of active targeting on the facilitated transport (*through Enhanced Permeation and Retention, EPR*) and the relative inability to attenuate the NPs bio-distribution. Several attempts have reported improved and enhanced NP tumor internalization so that the efficacy of their therapeutic payload is manifolded. It is pertinent to mention here that EPR involves the physicochemically induced intracellular drifting of drug-loaded

cargos and holds greater relevance than passive targeting. Permeation implies entry within the cells while retention refers to the residence of a drug inside the tumor cell.

- Studies aimed toward achieving enhanced therapeutic efficacy for actively targeted NPs are majorly prioritized toward the attainment of endosomal escape, partially accomplished by loading the drugs that are unreceptive to physiological destructive mechanisms (endosomal/lysosomal capture). For example, anti-HER2 targeting moieties conjugated on liposomal exterior aid in enhanced uptake for the HER-3 expressing tumor cells.

4.2 Factors Affecting Active Drug Targeting to Tumor Cells

- Ligand conjugation on a nanoparticle surface inevitably results in their altered properties, stemming from relatively distinct physicochemical activities. Yester investigations elucidate a key role of nanoparticle size (PS), geometry, surface charge, hydrophobicity, and composition in the stability of ligand-nanoparticle binding. The binding forces between the nanomaterial base and the corresponding ligands are of substantial importance and mandate precise monitoring so that the drug release at the needed site is facilitated via gradations in pH, temperature, and other physicochemical factors of the native cellular environment. The crucial concern herewith pertains to minimal structural degradation and consequent effects on native physiological events. Some specific biochemical conditions have already delivered splendid distinctions in nanoparticle-assisted drug delivery. For instance, one study revealed a significant opposition in the nuclease degradation for nucleic acid strands immobilized on NPs. The physicochemical parameters could be native or induced from the external end and therefore allow the researchers to look for multiple efficacies of a single drug-loaded NP. Select factors of eminence affecting the drug delivery ability of NPs through active mode are discussed in the subsequent paragraphs.
- **Distribution Intensity of the ligand**: Conjugation of a ligand on NP surface results in a trouncing of corresponding linear and translational degrees of freedom. As a result, the NP–ligand complex develops an increased reactivity with an enhanced valency. Owing to an enhanced valency, the substrate binding tendency of NPs is aggravated. On a thermodynamic scale, ligand binding with substrate catalyzes a henceforth loading of the neighbors. The net physiological outcome of such a cascade of reactions results in large-scale nanoparticle-cell membrane interactions, expressing finally as a clustered array of intense localized receptor actions. These changes fuel the tumor cell inside trafficking of drug-loaded NPs cargos arising from membrane wrapping-driven binding of normally low-affinity ligands with their explicit carriers at enhanced gluttony.
- Even though this mechanism enhances the ligand attraction and the ultimate cellular internalization, more ligand density is not always beneficial for a bettered intracellular drug delivery. Several investigations have reported rather limiting effects on cell binding as the ligand density increases above a threshold. Probable

factors attributing to this could be improper ligand orientation, steric hindrance of neighboring chemical functionalities, or competing for intramolecular tendencies for nearness to tumor cell surface receptor. Constraints of such nature are comprehensively reported in folic-acid targeted micellar-driven drug delivery characterized by patchy clusters of engineered ligands. Studies on cell line settings and live animal models for this configuration revealed a reduced receptor facilitated tumor cell internalization.

- Numerous erstwhile attempts also noticed an enhanced macrophage facilitated drug-loaded NPs uptake with the involvement of high-density hydrophobic ligands, rather exerting a limiting consequence concerning receptor-aided cellular internalization. Of significant interest are the outcomes of selected in vivo attempts, wherein engineered NPs surface (equipped with a binding large number of ligands) alter the blood circulation and bio-distribution of the drug-loaded cargos. A particular attempt in this regard used aptamer-driven delivery of polymeric NPs to prostate tumor cells and noticed that a 5% enhancement of ligand density resulted in spleen and liver-mediated systemic clearance owing to an abysmal tumor distribution. So, the ligand density of a drug-loaded NP must not be inadequately high or rather too low. On some occasions, relatively a different picture is observed wherein tumor cell-specific antibodies are forbidden to reach the tumor interstitium owing to the barrier marred binding site. A study noticing this reported the targeting of a 25 nm NP to epidermal growth factor (EGF) characterized by a little tumor penetration contrary to the nontargeted moieties. Contrary to this, a 60 nm-sized drug delivering NP was not affected and remained unaffected by this binding barrier, thereby illustrating the size-dependent tumor penetration of the drug-delivered NP cargos. Results of discussed studies herein underline a correct distribution of ligand density on NPs surfaces as a strategy to counter the binding-site limiting actions.
- **Variations in morphology and overall dimensionality**: No matter what is the context of NPs involvement in interactions, size and shape are the fundamental aspects regulating their physicochemical behavior and functional performance. Numerous investigations describe the shape- and size-dependent tumor cell internalization abilities of drug-loaded NPs. In one of these attempts, 5 nm Au-NPs were found unsuitable for drug delivery due to limiting ligand surface densities but surprisingly no such constraints were noticed for 15 nm sized particles. Readers must note here that conjugation of high molecular weight ligands to NPs surfaces often results in enhanced hydrodynamic radius that often impairs their tumor cell penetrating abilities. Optimum ligand orientation of drug-loaded NPs surface plays a decisive role in their stealth delivery and trafficking within tumor cells, as also confirmed in a study noticing greater intracellular retention for >50 nm NPs. Stealth delivery of drug-loaded NPs prevents their early elimination and prolongs their blood circulation. These observations were also supported by the studies of Lee and colleagues who noticed a higher tumor residence for (25 and 60) nm sized drug-loaded NPs contrary to that for 5 nm sized particles.

- Erstwhile investigations elaborate more on such distinctions, with enhanced uptake for (2–70) nm Au and Ag NPs conjugated with anti-Her2 antibodies, by the breast adenocarcinoma contrary to that for 25- to 50-nm-sized particles. On careful analysis, the investigators of this study found such distinctions as the outcomes of the strength of avidity and the extent of cell membrane wrapping around the NPs. Another important factor contributing to such artifacts was the dominance of surface interactions during the in vitro experiments (in the culture media), which impaired the transport within the tumor cells. These observations rule out a generalized perception that smaller-sized NPs are invariable better drug carriers and infer a normalization of multiple other quantum mechanical attributes before making a comparison concerning their size and drug delivery abilities. In terms of morphology too, several studies have reckoned a higher tumor cell intake and drug-delivering ability of rod and wire morphologies of NPs rather than those for spherical (Malik and Mukherje 2018).
- **Surface and ligand charge**: Charges on ligand and NPs surface do prevail in course of their formation and have a significant effect on their mutual interactions. So, conjugating ligand and nanoparticle with null charges is not feasible, however, the challenge is to moderate the ligand and nanoparticle binding via intervening neutral and inert molecules. Such approaches have been materialized but have irrespectively complicated cellular internalization due to higher sizes. A unanimous finding was the higher activity of cationic NPs which internalized efficiently within the tumor cells since the negative charge on plasma membrane lipids facilitates the electrostatic attractions. Even then, such interactions often result in the nonspecific prevalence of administered NPs. Though several studies focused on the dependence of charge density on the targeted NPs-cell interactions, yet best details to date do not wholesomely describe the parameters corresponding to maximum tumor targeting.
- **Hydrophobic/Hydrophilic sensitivity of surface**: Besides surface charge, dimensions, and morphology, hydrophobic sensitivity of drug-loaded NPs also plays a key role in the ultimate ligand orientation on NPs surface. Studies have noticed these distinctions for polymeric NPs, characterized by hydrophobic cores. In a distinguished attempt by Valencia and colleagues, it was demonstrated that trapping of folic acid (a hydrophilic periphery) within the Au-NPs core, resulted in its inadequate surface presentation (Fig. 16). As a result, the aggravated hydrophobicity of NPs surface resulted in its nonspecific cellular interactions.
- Such restrictions are countered due to missing steric stabilization at the NPs surface which forbids the ligand binding due to the interruption caused by surface adsorbed intracellular proteins. Thereby although hydrophobicity confers steric stabilization, studies reveal that too high hydrophobicity (longer alkyl chains) obstruct a ligand's accessibility to their respective targets. Such eventualities have been noticed squarely in the in vitro and in vivo attempts, exhibiting a substantial dependence on the average circulation time (within physiological barriers) of the carrier and the characteristic ligand-substrate interactions. Pressing compulsion herein is the loss of barely minimal polyethylene glycol (PEG) surface capping

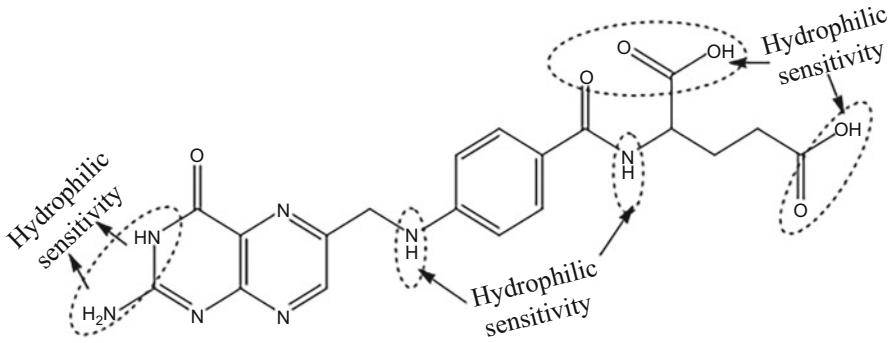

Fig. 16 Chemical structure of folic acid, depicting its plentiful hydrophilic substituents at different locations including terminal positions. These functionalities enhance the surface hydrophobicity of being masked within the Au-NPs core

(to prolong the circulation time and tumor distribution) of the drug-loaded NPs, in the close neighborhood of tumor cells.

4.3 Passive Targeting of Drug-Loaded Nanoparticles

- Contrary to the active targeting, the passively modulated targeted drug delivery of the NPs to tumor cells makes use of the concentration gradient for tumor cell penetration of the drug-loaded macromolecular complexes with the ligand conjugated NPs. Witnessed first before ~30 years, this approach relies on the enhanced permeation and retention (EPR) effect. The application of this phenomenon was noticed following the tumor internalization of poly(Styrene-co-Maleic Acid)-neo-Carzino Statin (SMANCS), a 16 kDa polymer conjugate. This giant molecule binds noncovalently to albumin, in course acquiring ~80 kDa molecular weight. Interest grew in exploring the biophysical interactions of SMANCS and progressive efforts revealed its preferential accumulation on account of imperfect blood vessels and poor lymphatic tissue conduit.
- Simultaneous permeation (entry) and longer stay within the tumor cells illustrate the EPR phenomenon, which is the backbone aspect of passive drug delivery principles. The following paragraphs are thereby devoted to understanding the EPR dynamics and its consequences in varied tumor microenvironments.
- EPR rationale establishes itself from the growth of a solid tumor corresponding to a characteristic size limit beyond which no oxygen is needed for its further proliferation. Owing to this, the tumor cells gradually perish and release the growth factors. These growth factors thereafter, resulting in the formation of newer blood vessels that arise from the surrounding capillaries. This newer blood vessel formation is termed angiogenesis and gradually results in the development of irregular blood vessels with concomitant participation of irregular epithelium. This irregular epithelium lacks the basal membrane, unlike the

conventional normal vascular structures. Inevitable consequences of these blood flow changes result in altered functioning of capillary physiology whereby sizes as large as 2000 nm are accomplished, depending on the specific tumor type, its location, and the typical microenvironment.

- This abnormal and irregular vascular arrangement can no longer oppose or restrain the trafficking of blood constituents to the tumor interstitium. This prospect comprises the enhanced permeation phase of the EPR effect. Contrary to their normal counterparts, tumor cells exhibit defective lymphatic drainage. Owing to this, the gradual discard and reformation of interstitial fluids are prevented, ruling out the revival of leaked solutes and colloids into the systemic circulation. This has a bearing particularly on macromolecules while those which are <10 nm in diameter are normally recovered through back diffusion within the blood circulation. Concomitantly, macromolecules remain unremoved from tumor interstitium and get accumulated, forming the retention domain of the EPR phenomenon.
- Ever since its first explanation and demonstration in the 1980s, EPR has been a formidable explanation for justifying the cellular residence within the tumor cells, about NPs-mediated drug delivery. As a characteristic developmental aspect, disturbed and irregular lymphatic functions in the tumor cells (particularly of the blood vessels in the periphery and the bulk) result in the bulk vessels' propagation under a considerably higher mechanical stress. As a result, the native functional distortion in the tumor cell interior regions is substantially greater than that in the marginal cells. So, a difference in the lymphatic integrity within a tumor cell is a prominent factor regulating the intracellular stay of drug-loaded NPs cargos.
- Although regular attempts and research efforts have significantly bettered the understanding of macromolecule distribution within a tumor cell, a significant element of concern pertains to a less number of EPR trials in humans. As a result, multiple concerns have restricted a wholesome illustration of EPR in many research investigations. A decisive factor contributing to such inadequacies is the dissimilarity in the factors required to optimize the retention and distribution in animals and humans. For example, the clearance principles and biomechanics of tumor cell functioning contrastingly differ for humans and animals. Further, the comprehensive studies of animal models subjected to NPs-mediated drug delivery in the preclinical attempts lack a predictive significance compared to those of humans. This is due to the much more complicated optimization of drug-loaded NPs in terms of their PCPs for humans as compared to the most preferred mice models. Finally, the local factors rule out a fair comparison of clinical attempts in humans and animals, such as that for PEGylated liposomal EPR, observed frequently in animals exhibiting an unusually high tumor to blood stoichiometry. Generalizations analogous to the above factors are rather inconsequential in humans, with only sarcomas as noted cancer models for a localized high liposomal accumulation and residence.

4.4 Factors Affecting the Passive Drug Targeting to Tumor Cells

- **Physicochemical Properties (PCPs)**: Among the most prominent factors regulating the biophysical interactions in distinct chemical environments, PCPs are the outcomes of characteristic bond energy, geometry, and vibrational extents of interacting molecules. Concerning NPs-mediated drug delivery, PCPs assume significance as the passive mode is aimed to deliver the drugs in the tumor vicinity from where these are expected to reach inside the tumor cells based on leaky vasculature and troubled extracellular matrix (ECM) constitution. Since the drugs delivered are bioactive molecules having their function as an explicit function of their native chemical structures, the interactions of delivered drugs with the carriers and the tumor cell-specific surface receptors must be moderate so that the inherent drug structure does not undergo any inadvertent loss in its native functional essence. As the physical state of delivered drugs and their chemical exposure concerning the colloidal state, the drug-loaded NPs must operate with controlled diffusivity and enhance the permeability of loaded drugs across the vascular separations. The success of an NPs-mediated drug delivery is intricately influenced by the PCPs which regulate the interactions with the immune cells that operate to detect them as foreign species and render their elimination from the physiological boundaries. The complexity of optimal PCP analysis is caused because of their dissimilar effect on blood circulation, the extravasation process, and the specific diffusion within the tumor cells.
- To maximize the tumor cell penetration and residence of drug-loaded NPs, an implicit parameter based on PCPs of drug-loaded NPs cargos is to ascertain the typical blood amount to which a drug-loaded NP is being exposed. Studies and investigations on animal models elucidate that a high blood concentration of drug-loaded NPs catalyzes its unidirectional diffusion across the tumor cells. Thereby, prolonged blood circulation of NPs-loaded drugs facilitates a larger tumor cell penetration of the loaded drugs. Readers must note here that the accumulation of soluble polymers inside the tumor cells infers its typical blood exposure in the conditions where the molecular weight of a polymer is enhanced to a value higher than the glomerular filtration threshold, whereby a subsequent polymer discard is prevented. Results of an investigation are worth mentioning here, wherein analysis revealed tumor accumulation upper limit for a macromolecule as inversely dependent on its renal exposure and a direct correlation with the total body exposure. In several other noteworthy attempts, tumor cell internalization periods for polymers, liposomes, and engineered NPs as drug carriers, have been determined and illustrated. An element of concern in this regard is the surface coatings on NPs (to prevent their immunological detection) which complicate their renal clearance and ultimately contribute to the intracellular physical stress.
- **Size**: The particle size (PS) or more familiarly, the hydrodynamic diameter of NPs-loaded drugs is a decisive factor regulating their kinetic activities and the explicit tumor cell internalization. Even though it is well documented that NPs of lower sizes are accumulated to a greater extent within the tumor cells, attaining a

low PS is highly challenging in terms of its lasting stability. This is because most of the preparation methods of NPs exhibit a residual charge on their surface and the size decreasing external forces face stiff opposition from the inward Laplace forces. Howsoever, shear is applied from the external end, it is almost impossible to reach the dimensions to an extent that remains unaffected by the influence of gravitation. These artifacts are substantially held in agreement by the studies on mice models, wherein 2- to 3-nm-sized NPs with 10 kDa molecular weight are not only accumulated to a higher extent within the tumor cells whereas larger counterparts with 70 kDa molecular weight were not screened as identically capable. Though lower-sized NPs initially accumulated to a larger extent within the tumor cells these diffused back in a vulnerable manner, from the vascular compartment. Another study demonstrates a critical role of NPs size in their distribution via modulating the EPR effect, wherein, unchanged circulation extents were observed for the varying sizes of NPs. This investigation noticed that the particles with <100 nm sizes were distributed in a comparable manner in the hyper-permeable tumors. On the other hand, the poorly permeable tumors exhibited an efficient accumulation of only the <70 nm sized NPs. Thereby, a direct relation seems workable between the tumor accumulation extent of drug-loaded NPs and tumor shrinking efficacy for studied drug-loaded platinum NPs. To summarize, this study explained the distinctions between blood circulation and tumor accumulating tendencies for <50 nm NPs.

- The prime conditions necessary to ensure a uniform and monodispersed size distribution of NPs pertain to a minimal existence of an aggregated state in the presence of dispersion stabilizers. Furthermore, it is now unanimous observation that a low PS is never a disadvantageous or negative factor for the drug delivery ability of a NP. A larger size may be developed due to self-assembly-driven interactions which arise due to synergistic molecular associations and the individual functional activity of each constituent is not tampered with. The stabilizing forces in such associations are weaker steric controls, regulated via London Dispersive and van der Waal forces of attraction. The paradox in this whole exercise is the characteristic tumor accumulation time for each NPs as it has a bearing on the corresponding toxicity induction and the extent of interactions with cell organelles and other constituents. Nevertheless, sustained toxicity induction is of course needed and is the working mechanism of most antitumor drugs.
- **Surface charge**: The surface charge of the drug-loaded NPs is another vital parameter that has a bearing on their systemic distribution and tumor cell-penetrating ability via modulating the EPR effect. A critical regulator of the stealth delivery of drug-loaded NPs, accounting for prompt elimination of the intercepted entities by the immune cells. The negatively charged plasma membrane (due to lipid bilayer) readily interacts with the positively charged drug-loaded NPs complexes by electrostatic interactions. These observations are strongly supported by the preclinical studies, wherein human model investigations exhibited greater suitability of cationic NPs on account of compatible interactive patterns for facilitated diffusion across the deeper regions of a tumor.

Readers are suggested to consult the more specific literature sources in this regard, focused on the uptake and intracellular accumulation behavior regulation control of drug-loaded NPs.

- The best illustration for a better performance of positively charged drug-loaded metal NPs as drug carriers is endowed concerning the explicit ionization extents, relative blood circulation profiles of control species, and the net charge distribution of drug-NPs assemblies. Several studies demonstrate greater tumor accumulation ability for sterically stabilized cationic dispersions. Nevertheless, the charged state of NPs–drug complexes is never free from instantaneous vulnerabilities, as also suggested by some investigations on ECM of mice sarcoma. These studies concluded excessive positive charge is a negative factor for the trafficking of drug-loaded NPs across the ECM, on account of the mutual electrostatic interactions (NPs surface charges and the ECM charged protein complexes/assemblies). These generalizations were further authenticated by in vivo inspections, demonstrating the tumor cell administration of neutral and charged drug-loaded NPs to mediate a sustained interaction of charged colloids with the tumors rather than the uncharged ones. Erstwhile in vitro attempts also support this argument, wherein masked NPs surface using high salt contents, like that of furnishing polyanionic proteins to a positively charged drug-loaded NP.
- **Shape**: Shape-dependent NP activities for their drug delivery attributes have been a topic of increasing attention of late, with the investigations revealing non-spherical morphologies as better. Studies screening the shape effect on NPs drug delivery response opine their key involvement in the detection and systemic removal by the immune system cells. In one such study conducted on Au nanostructures, Au NRs were noticed as capable of longer systemic circulation contrary to the spherical morphology. Analysis revealed a four-fold higher murine macrophage uptake of the spherical morphologies as compared to others. Further inspection of such shape-dependent macrophages' actions inferred a suppressed murine macrophage uptake on increasing aspect ratios (length to cross-sectional diameter ratio). These observations are supplemented by an erstwhile attempt, wherein four times greater extravasation was observed for 44-nm-long NRs contrary to the 35 nm nanospheres (NS) having similar R_H. Likewise, elongated viral nanoscale filaments obtained from plant viruses were screened to have a greater tumor residence than spherical morphologies. In another significant attempt, single-walled carbon nanotubes having an AR ranging between 400:1 to 500:1 were found to be systemically eliminated to a greater extent (regulated by kidneys) despite being 10 to 20 times filtering ability of glomerulus (100–500 nm). These observations collectively illustrate the higher suitability of elongated morphologies (higher surface area) for drug delivery due to their longer systemic prevalence and porous structures.

4.5 Common Factors Regulating Active and Passive Targeting

- The physiological dynamics of redox status in the tumor vicinity is a factor that commonly affects the active and passive targeting, as the delivered drugs are chemical structures and implicitly affected by modulation in the chemical environment. Furthermore, *apoptosis and necrosis* of tumor cells are always differently mediated with the former being less stringent while the latter mandates a drastic localized environment for altered physiology.
- Since the native environment of any tumor cell is always acidic, there is a significant probability that drugs coated with surface stability at >7 pH may be degraded rather too rapidly which may cause accumulated toxicity. So, there is a mandatory requirement that the surface coating of the being delivered drugs facilitates their sustained and gradual release and that's why it is important to test the in vitro stability of drug-loaded carriers before being evaluated in the in vivo environment. A summary of active and passive targeting is depicted in Fig. 17, wherein the targeting mechanism is highlighted as the distinction.
- Another factor of note is the mechanism of action of a characteristic anticancer drug. While the majority of these are equipped with a functioning that prompts apoptotic activation but this may not be possible at all stages of a developing tumor. In extreme cases or as is the usual scenario with most threatening cancers (lung and breast, for instance), the activation of apoptosis-inducing genes is biochemically infeasible, and thereby, cell death has to be induced deliberately. Such a chemical environment is drastic for normal cells propagating in the vicinity and owing to this; necrosis is the inevitable outcome of killing the cells.

4.6 Destruction of Cancer Cells by Altered Oxygen Levels

- Within a tumor cell, the NPs in native or anticancer drug functionalized textures exert several physiological responses as a result of which the oxidative balance within the tumor cell is altered. The crux of all anticancer treatments is either via scavenging of oxygen or via increasing its concentration to a considerably greater extent compared to the normal cells. Ultimately, the tumors are destroyed either due to lack of oxygen or via hyperoxia caused by oxidative stress.
- The conventional working model of standard chemotherapies is based on an antioxidant strategy in which oxygen-deprived cancer cells find it difficult to sustain themselves and ultimately perish. Due to this, the tumor cells of a particular tissue metastasize to newer and unaffected physiological locations driven via enhanced angiogenesis and permeable, heterogeneous tumor vasculature. Studies elucidating such dynamics suggest a vital role of oxygen lacking induced tumor cell starvation-induced metastasis (through Hypoxia Induction Factor (HIF) expressed on the cell surface) toward confronting the vigorous growth potential and persistence of human cancers.
- The altered oxygen content of a tumor cell affects its communication with the cells in the vicinity besides that with the endoplasmic reticulum and mitochondria

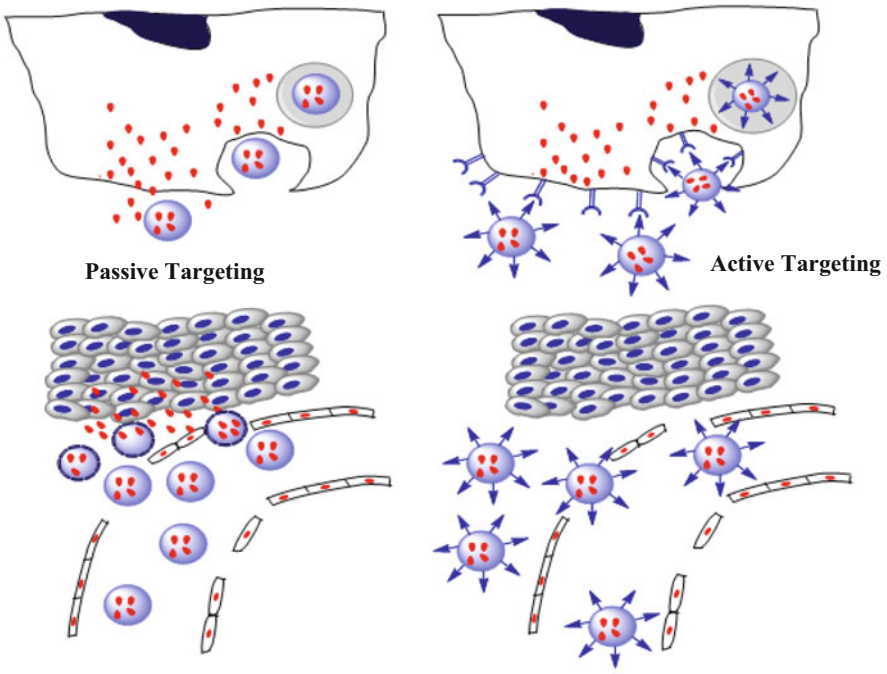

Fig. 17 Schematic representations of Passive and active drugs targeting tumor cells, with the former being manifested by circulating NPs which extravasate in solid tumor tissue by enhanced blood vessels permeability (unorganized and leaky vasculature surrounding the solid tumor coupled with an absence of lymphatic drainage, getting preferentially accumulated in tumor cells. In the active mode, on passive extravasation and concentration of NPs in the targeted tissue, the ligands grafted on nanoparticle surface facilitate active targeting of NPs to the receptors overexpressed on tumor cells, mediating enhanced uptake and internalization through receptor-mediated endocytosis. The figure demonstrates the release of the drug into the ECM followed by diffusion across cells and tissues (passive mode) and tumor-specific ligands on the NPs bind to the cell surface receptors to aid in the internalization of NPs via endosomes. Thereafter, due to internal acidic pH, the drug is released from the NPs and diffuses in the cytoplasm

to obtain energy for cellular actions. Studies exploring NPs drug-like actions in cancer cells unveil their anticancer response being regulated via enhanced intracellular oxygen, resulting in the oxygenation of tumor cell suborganelles. Based on this hypothesis, the loading of drugs on the ligand conjugated NPs is programmed in a manner to enhance this intracellular oxidative stress-driven cell death. This implies that drugs killing tumor cells via necrosis or enhanced oxidative stress are likely to perform better if these are delivered with NPs, as the possibilities of synergistic response are higher.

- Dissimilarities in conventional chemotherapy and NPs-mediated drug delivery to cancer cells are based on comparatively high instantaneous intracellular toxicity in the former contrary to a sustained response (relying more on apoptosis). This infers that delivery of chemotherapeutic drugs using NPs mediates their sustained

response in a kinetically escalated manner whereby the risk of instantaneous aggravated toxicity is prevented. Although NPs do contributes to this mode of drug delivery but their alone actions are always less pronounced compared to those of NPs loaded with chemotherapeutic drugs. Further, the selection of NPs for sustained action of chemotherapeutic drugs is indeed crucial as only those NPs which act via catalyzing apoptotic induction (chemically very less reactive) are more suited to control the action via the antioxidant route. For example, Au-NPs are themselves very inert, so delivering drugs via loading on Au-NPs operates more by the mechanism of chemotherapeutic drugs while those delivered using Ag NPs as carriers derive their toxicity partially from the toxic response of Ag NPs, the mechanism for which is well-documented in several reports.

- In a nutshell, the anticancer actions of Au or Ag NPs are controlled via interfered enzymatic activity, altered actions of intracellular communication sustaining protein channels, or via inducing mutations in DNA base pairs of cancer cells. Although it is possible to tune the working mechanism of NPs-mediated drug delivery via antioxidant or pro-oxidant route, the majority of NPs-mediated drug delivery attempts make use of enhanced oxidative stress by stimulating the intracellular reactive oxygen species (ROS) actions. Readers are suggested to understand such dynamics by learning about the implicit working mechanisms of various chemotherapeutic drugs in their alone delivery and differential tuning by active and passive drug delivery using NPs.

5 Recent Attempts of Gold and Silver Nanoparticle-Mediated Drug Delivery to Lung and Breast Cancers

5.1 Lung Cancer

- The best literature analysis to date, reveals the A549 cell line as the mostly employed lung cancer (LC) cell line in use for analyzing the drug delivery efficacy with various NPs. Screening of NPs drug delivery efficacy in the anticancer cells has been made as a function of varied sizes and shapes which are the implicit parameters affecting the quantum mechanical characteristics. These parameters also have a key role in regulating the toxicity of drug-delivering NPs. Recent attempts of Ag NPs-mediated drug delivery to lung cancers suggest their highly vibrant anticancer activities via modulated intracellular redox balances. A common aspect of nearly all attempts remains the optimizations to accomplish enhanced tumor cell internalization by tuning the EPR effect. Interestingly, almost every study reports a positive influence of assisted photo and thermal response to accomplish significant anticancer effects, analyzed via changed pro and antiapoptotic protein expressions. Despite similar sizes, preparation methods, and stabilizing agents in use, the constitutional arrangement of electrons in Au and Ag is responsible for the higher toxicity of Ag NPs. This is well-supported by the $5s^1$ and $6s^1$ valence shell electrons of Ag and Au, making the Au^+ formation easier as less energy is required to remove an electron from the

farther 6 s subshell. Thereby, Ag^+ formation from Ag requires greater energy from outside, making Ag^+ more toxic than Au. Following paragraphs describe the select studies from 2018 onward, the listing of which has been made via including major work while accompanied references could be searched in the main article referred due to the publisher instructions of not including more than 50 references.

5.2 Anticancer Therapies Using Gold Nanoparticles for Lung Cancer

- Commencing in 2018, the first noticeable attempt regarding Au-NPs-mediated LC treatment comprised a study from China, wherein the investigators prepared Au nanospheres (NS) using $HAuCl_4$ as a precursor. Hydrazine monohydrate and Bovine serum albumin (BSA) were used as reducing and stabilizing agents, respectively. The method enabled the formation of 10 nm, positively charged Au NS with a single crystal morphology which was thereafter, loaded with gemcitabine (GEM) and evaluated against the A549 LC cell line. Analysis of anticancer activities for neat and GEM-loaded Au NS revealed unloaded Au NS as biocompatible moieties with much lower cytotoxicity and significant anticancer potential. Thereby, the study demonstrated a green method for steadfast Au NS preparation which attributed well to a synergistic response on being loaded with GEM and exerted a tumor cell-specific toxic response (Wang et al. 2018).
- A nearly similar research attempt aimed toward optimizing the site-specific actions of Au nanoclusters (NC) using cationic BSA stabilization. Of note, the investigators noted enhanced functional efficacy after hyaluronic acid (HA) conjugation. Variation of BSA functionalized Au NC and the conjugated HA stoichiometries provided 100, 200, and 300 nm sizes of the integrated nanoscale complex. Inspection of as-formed drug-loaded nanocomplexes for cellular retention revealed insignificant distinctions for 100 and 200 nm sizes by the RAW246.7 and 4 T1 tumor cells. The distinctions in size-dependent response revealed easier MPS sequestration of 300 nm with low in vivo circulation duration. To minimize the size-dependent risk of inadvertent toxicity, 200 nm NC was chosen to load paclitaxel as an anticancer drug and indocyanine as a contrast agent in the integrated nanocomplex.
- Extensive characterization of surface features, morphology identification, and cytotoxicity induction revealed a higher cellular residence of HA conjugated Au NCs compared to those without HA conjugation. These observations were complemented by higher cytotoxicity of non-HA-conjugated NCs. So, the manifold –OH groups of HA moderated the BSA functionalized Au NC hydrophobicity and compensated the hydrophobically stabilized interactions with philophobic force gradients.
- These interactions were moderate and did not damage the hydrophobically rich chemical structures of paclitaxel and ICG. Deliberate selection of 200 nm NCs for drug delivery might have been made to minimize the risk of accidental

nonspecific interactions with 100 nm sized NCs. Intracellular accumulation of 200 nm NCs inferred a role of HA and BSA (cationic) electrostatic interactions in stabilizing the Au NCs. An important observation of this study is the inability of trafficking >100 nm sized NC within the tumor cells even by EPR but this was resolved by HA-mediated capture of BSA functionalized drug complex, which enhanced the permeability of drug-loaded NPs conjugate. Overexpression of CD44 as the membrane protein of tumors provided a structural inference of the binding site which facilitated the positively charged HA functionalized drug complex targeting. Results fall in agreement with the cancer cell hyper-permeability and nonfunctional backflow through irregular lymphatic drainage resulting in enhanced interstitial fluid pressure to avoid a convective transport and passive diffusion of NPs.

- Another significant attempt to make use of Au-NPs drug transport ability, used them in free and liposome embedded configurations for the delivery of temozolomide (TMZ) in a liquid state. The NPs were loaded with TMZ in a free as well as liposomal encapsulated state, after which the respective anticancer potentials were screened in the urethane-induced LC in BALB/c mice. Dispersion analysis of Au-NPs in free and liposomal bound configurations revealed a uniform distribution with spherical morphology. Screening of LC treatment efficacy was made via considerably reduced lipid peroxidation and inflammatory cytokine expression with enhanced glutathione activities. Analysis revealed TMZ-loaded Au-NPs as better antioxidant entities via modulated glutathione actions besides suppressed lipid peroxidation. Yet again, this study elucidates a synergistic cytotoxicity induction of TMZ and liposomal encapsulated Au-NPs, which contribute to an additive influence and aid inhomogeneous distribution of the loaded drugs with enhanced residence time in the tumor cells (Hamzawy et al. 2018).
- Another significant 2018 effort dedicated to using Au-NPs as drug carriers for LC treatment included an attempt from China, wherein electrostatically stabilized fluorescent Au NC conjugated chitosan and nucleolin targeting AS1411 aptamer was loaded with methotrexate. The drug conjugated Au NC was stabilized via strong hydrophobic interactions and exhibited 200 nm as particle size with 13.8% drug loading ability and a pH modulated drug release ability. Analysis for cellular uptake and anticancer efficacy was made using A549 cell lines, wherein selective uptake and >50% anticancer response was noticed. Subsequently, the drug delivery efficacy was analyzed via intravenous administration into BALB/c mice, which exhibited a significant decline in tumor growth on methotrexate accumulation with moderate cytotoxicity. Chitosan stabilization and nucleolin targeting of the as-prepared Au NC were screened as significant modulators of site-specific response, making the Au NC suitable for clinical attempts aimed at reduced A549 cell line multidrug resistance (Guo et al. 2018).
- Another 2018 glory exploring the suitability of Au nanocarriers as reliable drug carriers for LC treatment is the study of Ramalingam and colleagues, wherein polyvinylpyrrolidone (PVP) functionalized Au-NPs were loaded with doxorubicin. Cytotoxicity analysis of unaided and nanocarrier-aided doxorubicin delivery

revealed greater cytotoxicity for PVP-functionalized Au-NPs-mediated doxorubicin delivery, accompanied by a considerable suppression of A549 LC cell line proliferation. Cautious analysis inferred the ROS enhancement-driven anticancer response, supplemented by the sensitized mitochondrial membrane potential along with induction of late and early apoptosis. The study thus established the utility of PVP as biocompatible drug delivery supporting polymer toward accomplishing an improved doxorubicin delivery using Au-NPs as carriers which engineered an enhanced tumor cell internalization of drug-loaded nanocarrier (Ramalingam et al. 2018).

- The next significant 2018 attempt is the study conducted by Nam and colleagues, which explored the synergistic photo and chemotherapeutic responses to channel the cytotoxicity of polydopamine-coated spiky Au-NPs through its photothermal ability. Analysis revealed splendid photothermal activity of the prepared Au nanoassemblies, with single photothermal therapy exposure resulting in restricting the doxorubicin toxicity in a localized environment in >85% animal models. The study fundamentally monitored the growth of CT26 colon cancers but a considerable therapeutic response was observed in TC-1 submucosal LC, an aggressive metastatic form of head and neck squamous cell carcinoma. Supporting the anticancer response were the reductions in TC-1 metastasis to the lungs (Nam et al. 2018).
- In the last noted 2018 research, efforts used cyclodextrin (CD: α, β, and γ) variably functionalized with Au-NPs, that were stabilized via PEG coating. Following CD conjugation of PEG-coated Au-NPs, these were encapsulated with curcumin after which characterization for dispersion analysis was done using PS measurements. Inspection revealed curcumin-CD-Au-NPs combine to have 5 nm of interior diameter (screened only for Au-NPs) with 25 to 35 nm as average PS for the chosen CD grades. Screening the in vitro cytotoxicity of curcumin-loaded Au nanocarriers on the A549 LC cell line, the investigators noticed significant toxicity corresponding to all CD grades contrary to Au-NPs conjugated with CD and curcumin (in solution mode). These distinctions in cytotoxicity responses inferred the cytotoxic response as an outcome of the combined actions of Au-NPs and curcumin. Thermal conduction sensitivity of Au-NPs proved significant in their anticancer response due to heat energy-driven sustained structural oscillations in curcumin that contributed to the anticancer essence of curcumin-Au-NPs combined nanocarrier (Hoshikawa et al. 2018).
- Several studies also focused on the combined anticancer potency of NPs and energy in the form of heat, in combinative mode. Though temperature has a risk to affect the delivered drug structure a rigorous optimization can facilitate slow and gradual assistance toward localized drug activity. Realizing these abilities, a 2019 study from Korea-loaded ginsenoside (the drug) on the Au-NPs which were earlier prepared by the same group using probiotic *Lactobacillus kimchicus* DCY51, isolated from *Korean kimchi*. The drug-loaded NPs were rigorously screened for their structural compatibility using TEM, energy dispersive X-ray spectroscopy, X-ray powder diffraction, Fourier-transform infrared spectroscopy (FT-IR), and dynamic light scattering (DLS). The analysis inferred a noncovalent

drug binding with the NPs after which the loading extent was determined using liquid chromatography-mass spectrometry (LC-MS). The biologically prepared NPs developed no aggregation in the blank and drug-loaded configuration, as it is the frequent observation of varying the pH and ionic environment. Delivery of drug-loaded NPs in combination with laser treatment to A549 human lung cancer cells revealed greater apoptosis compared to that of only drug-loaded NPs. So, this study elucidated a *photo-conducting sensitivity of biologically prepared NPs to express synergistic chemotherapeutic effects* in lung cancer cells. The outcomes of this investigation could be extended to chemically and physically prepare Au-NPs in drug-loaded configurations so that a comparative analysis could be made concerning stable drug binding and synergism with photothermal activities (Kim et al. 2019).

- Another 2019 attempt revealed significant improvement in cisplatin chemotherapeutic efficacy through its sustained delivery upon being coated over the Au-NPs, which were prepared separately using Turkevich's method. The as-prepared NPs were stabilized in 3-layer mode using phosphatidylcholine, hexadecane thiol, and high-density lipoprotein. Thereafter, keeping in mind the vascular density-driven heterogeneity in the distribution of three-layered Au-NPs in tumor tissue and subsequent drug release, the computational simulations were applied. The model parameters were fixed using experimental measurements with 2D and 3D cultures of A549, H358, and PV9 nonsmall cell lung cancer cells. Before cisplatin loading, the as-prepared Au-NPs were thoroughly characterized using ζ-potential measurements (determined as -6 mV), hydrodynamic size determination (found as 80.2 ± 12.4 nm), shape and size determination using Scanning Electron Microscopy, and confirmation of lipids on the particle cores using FTIR. The cisplatin loading efficacy was screened as $78.9\% \pm 0.7\%$, while the release extents were $59.1\% \pm 2\%$ cisplatin in the initial 3 h, $76.7\% \pm 1.84\%$ cisplatin within 48 h, and $78.9\% \pm 2.1\%$ cisplatin within 96 h in the initial 3 h. These observations suggested the success of NPs aided cisplatin delivery for its sustained and long-lasting effect, thereby moderating the toxicity generated in unaided delivery. The observations were adequately supported by the decreasing tumor regression observed through sequential increments of postdelivery duration. The study analyzed the drug effect in different tumor regions based on oxygen availability and utilization, concluding in terms of *comparative extravasation*, and was in fact, the first major attempt toward quantifying drug-loaded NPs response in tumor cells using *vascular network-induced tissue heterogeneity* (Miller and Frieboes 2019).
- Moving on to 2020, multiple studies focused on NPs-mediated drug delivery to LC, the first attempt comprised an investigation from South Africa, wherein Crous and Abrahamse reported an improved response of photodynamic therapy targeted to eliminate cancer stem cells (CSCs) through its combinatorial application with Au-NPs. The study involved the preparation of a nanobioconjugate (NBC), using a photosensitizer, $AlPcS_4Cl$, Au-NPs, and antibodies, Abs. The as-prepared NBC was characterized using different spectroscopic techniques and the photodynamic effects on the lung CSCs, which were done via biochemical

assays post 24 h of irradiation. Analysis revealed a structurally compatible $AlPcS_4Cl$, Au-NPs, and Abs binding with moderate interaction forces (in an in vitro setting), which attributed to the *stability and significant photodynamic* activity of NBC. The NBC was noticed to be efficiently internalized in the organs regulating cell homeostasis. To evaluate the photodynamic effect, the various biochemical assays were analyzed after 24-h irradiation of LC cells with the NBC. Inspection revealed an enhanced localized toxicity expression with a significant cell death extent when the $AlPcS_4Cl$ was conjugated with Abs and Au-NPs compared to the $AlPcS_4Cl$ alone. Enhanced CSC death upon a photodynamic treatment inferred a significant anticancer response of Au-NPs aided photodynamic treatment, conveying an additive association (Crous and Abrahamse 2020).

- The subsequent 2020 attempt used a combination of chemo, radio, and thermal therapies of Au-NPs to treat A549 LC cells. The study from China used hollow Au-NPs (HGNPs) conjugated with polyoxyethylene sorbitol oleate (PSO, as a ligand) owing to its efficient recognition by the low-density lipoprotein (LDL) receptor that is overexpressed on A549 LC cells. Following this, a PSO modified doxorubicin-loaded HGNPs were designed and characterized for their combinatorial therapeutic activities, intracellular uptake, and the escaping detection from macrophage-assisted phagocytosis studied in vitro in the A549 cells. After in vitro analysis, the doxorubicin-loaded HGNPs were also targeted to LC in a mouse model. Significant therapeutic activity was observed, arguing well for a PSO, HGNPs, and doxorubicin synergistic response, with merely 10% cell viability at 500 μM extent. The uptake mode of drug-loaded HGNPS was identified as receptor (here LDL) mediated endocytosis with an ability to *escape macrophage detection*, together leading to an enhanced accumulation at the target site. The in vivo studies in this investigation encourage an inspection further through clinical trials, due to higher reliability than in vitro studies (Shen et al. 2020).
- Another promising attempt from China used an Au nanorod engineered using BSA and subsequently coated with RGD peptide and internalized with neutrophil, to finally design a neutrophil-based drug delivery vehicle. Engineered neutrophils (ENs) showed efficient migration across the epithelial cells, on account of inflammatory signal detection. The ENs exerted a higher toxic response against Lewis cells in the presence of laser irradiation in an in vitro model. Neutrophils acted as efficient cell carriers as, without their conjugation, the drug-loaded carrier the tumor tissue targeting ability in Lewis tumor-bearing mice was reduced by a significant extent. The results together concluded that a neutrophil-aided localization of BSA and RGD peptide conjugated Au nanorods to the LC cells for a possibility of deeper diffusion within the tumor cells, together resulting in inhibited tumor growth and more than 120 days of additional survival of the sufferer. Thereby, this attempt also proved a successful combination of Au-NPs with photodynamic therapy for enhanced therapeutic activity. The conjugation of *RGD peptide, BSA protein, and neutrophil receptors* are a vital

database that could improve the subsequent attempts for an improved response (Ye et al. 2020).

- The last noticeable 2020 attempt involving Au-NPs-treated LC used targeted Au-NPs in combination with clinical radiation therapy to screen the dynamic changes in tumor hypoxia. The study reported from the United States, gather encouragement from the previous attempts of investigators wherein specific tumor vascular disruption in mouse models of LC was reported using endothelial targeted Au-NPs in combination with external beam irradiation. The reduced tumor growth was assessed via *noninvasive whole-body fluorescence imaging*, whereby tumor hypoxia was examined at the basal level, after 2 and 13 days after tumor vascular disruption. After 2 days of combined therapy administration, a 2.5-fold enhancement in tumor hyperoxia was noticed but it resolved 13th day onward. It was noticed that a combination of vascular targeted Au-NPs and radiation therapy caused significant tumor suppression, making the analysis the first one to demonstrate the postdrug delivery tumor hypoxic physiological response and recovery. The study also analyzed tumor growth variations in the human nonsmall cell lung cancer athymic Foxn1 mouse model, yet again making it authentic to be examined via clinical trials (Virani et al. 2020).

  Thus, Au-NPs could be utilized as anticancer agents in three functionally distinct configurations. The first one is their inherent photothermal attributes which express in synergism with externally conferred chemotherapies. The second one is a *combined diagnostic and therapeutic mode* (theranostic approach) in which Au-NPs bound with fluorescent active molecules readily show changes after meeting abrupt tissue densities (in tumor cells), this stimulus is linked with needed drug release at the very same moment an abrupt tissue is sensed. The third one is through their native nanoscale architecture that facilitates an expression of enhanced binding sites via multifold SA increments.

5.3 Silver Nanoparticles for Lung Cancer Treatment

- The lone significant study of 2018 reported a preferential anticancer activity of Ag NPs prepared using *Garcinia mangostana* bark extract in combination with ultrasound treatment through their delivery to human lung epithelial cells. The NPs-ultrasound combine was screened efficiently toward preferential destruction of cancerous cells. Analysis of ultrasound and Ag NPs exposure on the A549 cancer cell line and the healthy BEAS-2b lung cells using flow cytometry revealed a minor decline in the count of cells (from 66.8 ± 3.2 to 56 ± 6.2)%, on adding of bark extract-driven Ag NPs to ultrasound treated normal BEAS-2B cell lines. Distinctly, the live A549 cells developed a suppressed count from (61.4 ± 4.2)% for ultrasound-treated cells to (28.7 ± 6.4)%, for being treated with an ultrasound and Ag NPs combination. So, this investigation established the mechanistic basis of Ag NPs-ultrasound combination efficacy for targeted destruction of cancer cells (Zhang and Xiao 2018).

- Among the manifold 2019 attempts using Ag NPs for NSCLC treatment, the first one was aimed at making an electrochemical nanobiosensor aimed toward screening Gemcitabine (GEM) interaction with EGFR exon 21-point mutant gene. The sensor assembly was designed using a new molecularly bioimprinted siloxane polymer (MBIS), characterized by the interaction of exon 21 with the identification probe. The functional sensor configuration used Ag NPs and MWCNTs as signal amplifiers. MBIS film in the sensor was prepared through acid-catalyzed hydrolysis of a solution containing Ag NPs, double-stranded (ds) DNA of EGFR exon 21 point mutant gene, GEM as a template molecule, 3-(aminopropyl) trimethyl siloxane (APTMS) and tetraethoxysilane. The GEM and double-stranded DNA (of EGFR) interaction was ascertained via monitoring the guanine and adenine oxidation response signals. Analysis of the developed sensor configuration using XRD, FE-SEM, EDS, FT-IR, and differential pulse voltammetry (DPV), revealed the corresponding oxidation signals to be in the linear range upon subjecting the device with 1.5 to 93 μM ranged GEM concentrations. The low detection limits of 12.5 and 48.8 nmol•L^{-1} were recorded for guanine and adenine respectively. So, this study reported an effort to examine the efficacy of *MWCNTs engineered Ag NPs* as efficient and accurate sensing moieties for ascertaining the GEM antitumor efficacy. The results could be bettered using other than MWCNTs signal amplifiers with Ag NPs (Shoja et al. 2019).
- Another significant 2019 attempt evaluated a combined action of shikonin and Ag NPs for apoptosis in A549 human NSCLC cells. It is pertinent to mention here that shikonin is a major constituent of zicao (the dried root of *Lithospermum erythrohizon*), Chinese herbal medicine with manifold biological activities, including inhibition of human immunodeficiency type-1 virus (HIV-1). Chemically, the R-enantiomer of alkannin, shikonin comprises *multiple –OH groups and double bonded oxygen* with distributed double bonds and diffused pi-conjugation, similar to several other polyphenols (Fig. 18).
- In their study, the investigators used shikonin as a reducing and capping agent for the green synthesis of Ag NPs. To screen the synergistic therapeutic response with Ag NPs, the radiolabeling of shikonin stabilized Ag NPs was conducted for intracellular tracking. The anticancer effect of shikonin capped Ag NPs was studied using tissue culture assay, wherein IC_{50} on the 24-h treatment of A549 cells using the MTT assay was screened as 2.4 ± 0.11 μg•mL^{-1}. Thereby, this study augmented the therapeutic activity of Ag NPs using *sustainable and biocompatible* shikonin capping. Results could be extended to other NPs and structurally similar to shikonin drugs for an additive response (Fayez et al. 2020).
- Yet another milestone attempt from 2019 examined the effect of Ag NPs in combination with ionizing radiations on mitochondrial redox state and functioning in *A549*, *BEAS-2B*, and *Calu-1* cell lines. The justification provided by the investigators in support of their mitochondrial functioning investigation was that the mitochondria are a powerhouse of a cell and are vitally involved in ATP (the energy currency of a cell) and reactive oxygen species (ROS) generation. The altered stress levels are routinely dealt with by mitochondrial antioxidants but once the stress levels are significantly higher, the damage is reported in

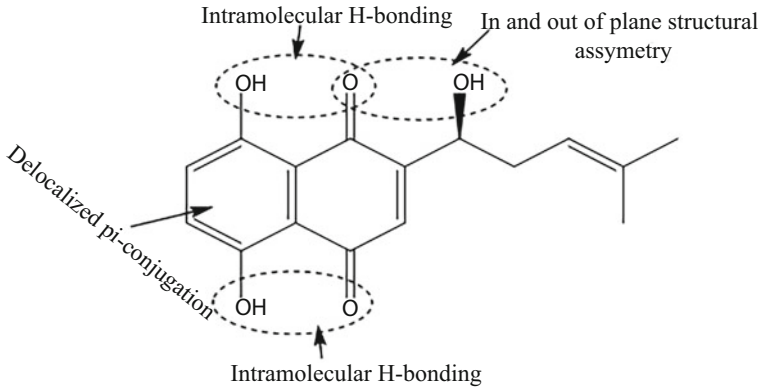

Fig. 18 Chemical structure of alkannin, of which shikonin is the R-enantiomer. The presence of multiple –OH and double bonded oxygen in this compound confer manifold biological properties, ranging from anti-inflammatory, antioxidant, and anticancer

mitochondrial DNA, proteins, and lipids. Mitochondrial antioxidant defense is regulated by protein cysteine thiols, the modification of which comprises an integral component of redox signal transduction and mitochondrial-cell communication. The investigators further stated that estimation of sulfenic acids is an implicit indication of monitoring protein oxidation in the manifold cellular processes. The rationale for studying mitochondrial effects of Ag NPs exposure has been suggested because the lungs are the first organs to gain access to external materials following inhalation. It is therefore of utmost importance to study the sublethal effects of Ag NPs and radiations on mitochondria. Analysis of mitochondrial damage on the chosen LC cell lines resulted in cell cycle arrest and decreased cell proliferation in A549, BEAS-2B, and Calu-1 cell lines but not in the NCI-H358 cell line. Increments in the expressions of mitochondrial reactive oxygen species (ROS) and protein oxidation were noticed as a function of time and dosage in more sensitive cell lines after Ag NPs exposure but not in the NCI-H358 cell line. It is pertinent to mention here that among the chosen cell lines, A549, Calu-1, and NCI-H358 were cancer cell lines but the BEAS-2B cell line is a transformed lung epithelial cell line. For the Ag NPs toxicity study, the researchers took note of their earlier observations of *size, shape, and coating independent toxicity* impacts in TNBC cell lines and therefore, used the Ag NPs with a minimal PVP coating (0.2 wt%). As used Ag NPs were spherical, 23 ± 14 nm in size, and exhibited -36 mV as ζ-potential at a pH of 7. Since the particles existed in a heterogeneous state of varying-sized aggregates on being dispersed in media, therefore for the experiments, the cells were exposed to Ag NPs in a complete medium. An important factor that could be the reason for the distinct toxicity of exposed Ag NPs in the studied cell lines was their dissimilar uptake abilities, with the more sensitive Calu-1 and BEAS-2B exhibiting higher Ag NPs intracellular extents contrary to A549 and NCI-H358 cells. So, this study explained the toxicity mechanisms of Ag NPs in the respiratory cells, with the

source being the *enhanced ROS activities through altered mitochondrial functions* (Holmila et al. 2019).

- In the subsequent 2019 study which was an Indo-Chinese collaboration attempt, the biomedical investigation of fine characterized Ag NPs prepared using *Gossypium hirsutum* (cotton) leaf extract, focusing on the molecular mechanisms involved in the activation of mitochondrial-mediated signaling pathway by the Ag NPs in A549 human LC cells. It was observed that on being treated with Ag NPs, the A549 cells exhibited G2/M arrest, ultimately leading to apoptosis-driven cell death. This observation was complemented by the suppressed expression of antiapoptotic (Bcl-2) and enhanced expression of proapoptotic (Bax) mitochondrial genes. It was further examined that these altered gene expression patterns may interrupt a native mitochondrial membrane potential, facilitating the release of cytochrome c into the cytosol. This release of cytochrome c activates caspases 3 and 9, which are responsible for intrinsic apoptosis signaling-driven cell death induction. A further analysis inferred no other harms of administered Ag NPs, including *swelling and inflammation* in mice organs. On the whole, this investigation established the mechanistic basis for cotton crop synthesized Ag NPs induced cell death via apoptotic induction (Kanipandian et al. 2019). The results of this study could be extended to crops similar to cotton growth patterns and requiring similar nutrients at different growth stages.
- Significance of interest toward NPs enabled drug delivery could be anticipated from the 2020 study, which used biomolecules functionalized core-shell configuration of magnetic nanocomposite (NC). The NC comprised of *Ag NPs functionalized with biguanidine–chitosan combination in a core-shell regime*, functioned analogously to using polysaccharide materials as potential coating agents to stabilize the metal NPs. The as-generated magnetic NC was rigorously characterized using ICP, FE-SEM, EDX, HR-TEM, VSM, and XRD. The investigators observed the as-formed NC be effective in the *multicomponent synthesis of propargyl amines* (PGA) using A^3 coupling in a hydrophilic environment. The use of NC provided extraordinary PGA yields. Further due to its strong paramagnetism, the investigators used this catalyst for biological assays, such as antioxidant activity monitoring using DPPH free radical assay (with BHT as reference). After obtaining a significant IC_{50} extent in the radical scavenging assay, the anticancer attributes of catalyst were studied in PC-14, LC-2/AD, and HLC-1 human LC cell lines. Analysis revealed the best activity in the PC-14 cell line with minimal IC_{50} extents, thereby exploring the structure–activity relationship (SAR) of fabricated NC. Results convey a need to test the prepared NC through a variation in their magnetic response and in synergism with multiple capping agents, which could improve the response and be useful for future attempts (Ma et al. 2020).

5.4 Gold Nanoparticles for Breast Cancer Treatment

- Affecting mankind for the past several years, breast cancer (BC) is the most prevalent menace in females across the globe. The conventional medications and therapies have only been able to reduce the troubles through a curative approach. One of the crucial bottlenecks implicated in this is the delayed identification of cancer, mostly at the time when the tumor has spread to multiple organs. The metastasis of BC to the lungs is the most noticed to date. Besides delayed identification, recurring multidrug resistance to conventional medications has posed significant hurdles in their activities. Recent attempts on Au-NPs-mediated drug delivery to BC are widely optimized through *shape selective thermal and structural activities*.
- Several 2018 investigations examined the BC treatment ability of Au-NPs, wherein the very first attempt by Calavia and colleagues used photon and thermally sensitive Au-NPs. The prepared NPs used sodium borohydride as a reducing agent after which their functionalization was done via mixed monolayers of PEG and the varying alkyl chain possessing zinc phthalocyanines (ZnPcs). The two ZnPcs differed in the length of carbon chains connecting the phthalocyanines to the Au core. Of the two photosensitizers, the one with 11 carbon atoms exhibited higher fluorescence emission intensity. A surprising observation was the higher fluorescence of shorter carbon chain phthalocyanines when it was bound to the surface of Au-NPs. A similar observation was made for the generation of singlet oxygen ($^1O_2$). So, this study provided significant inputs about the optimization of fluorescence emission with varied hydrophobicity. Screening the anticancer efficacy, the investigators observed that metal-enhanced $^1O_2$ generation contributed to enhanced photodynamic performance which proved effective in the treatment of human BC cells (Calavia et al. 2018).
- The subsequent 2018 study using Au-NPs for BC treatment examined the doxorubicin delivery by encapsulating solid and hollow Au-NPs, adsorbed over the thermosensitive liposomes (TLs). The investigators noticed nanoscale switches-like actions of solid and hollow NPs morphologies which catalyzed the doxorubicin release in the BC cells. The as-prepared NPs were screened for their photothermal and chemotherapeutic efficacy, where the hollow NPs emerged as superior for the doxorubicin uptake besides exhibiting lower toxicity compared to those of solid NPs. The drug-loaded hollow NPs exhibited a spherical morphology with −29 mV ζ-potential and eight times greater anticancer potential compared to the solid NPs. One possible reason for such a distinctive response was suggested as the greater hypothermic capability of hollow NPs that resulted in the conversion of an incident near-infrared (NIR) light into heat at the targeted site and triggered the doxorubicin release from the drug-loaded nanovehicle (Li et al. 2018).
- Another prominent research attempt from 2018 employed Au and Ag NPs prepared using *Phoenix dactylifera* pollen extract. The as-prepared NPs were thereafter characterized for their structural integrity and the *Phoenix dactylifera* phytoingredients quantification (Banu et al. 2018). Screening the toxicity of

as-prepared NPs on MCF-7 BC lines revealed a dose-dependent cell death, monitored using fluorescent microscopy and the expression of p53 (proapoptotic) and Bcl2 (antiapoptotic) proteins. The 2.57 and 1.64 increments and decrements in p53 and Bcl-2 expressions were noticed following treatment with Au-NPs, while the Ag NPs were screened as less effective with 1.40 fold aggravated p53 and 1.08 fold decreased Bcl-2 expressions. It is pertinent to recall here that p53 is a proapoptotic protein, the transcriptional activation of which is directly related to apoptotic initiation. This protein also modulates the expression intensity of Fas and Bax, two prominent apoptosis-associated proteins. Likewise, increased Bcl-2 functioning caused a reduction in mitochondrial membrane permeability and catalyzed the release of proapoptosis aggravating molecules, thereby delaying the mitochondrial membrane permeability. This induced the release of pro-apoptotic molecules that reduced the intrinsic mitochondrial regulated cell death. Thus, both Au and Ag NPs induced cancer cell death by altering the expression patterns of apoptosis-related p53 and Bcl-2 genes.

- Another significant 2018 attempt used *tumor cell-derived extracellular vesicles (TDEV)* (encouraged by their natural formation and cargo delivery abilities) constituted nanoplatforms for multimodal miRNA delivery, photothermal therapy administration, and magnetic resonance imaging (MRI) of 4T1 BC cells. The study reported from Stanford University featured in ACS nano-loaded anti-miR-21 to the carrier vehicle which blocked the function of overexpressed endogenous oncogenic miR-21 in 4T1 cells. The investigators designed Cy5anti-miR-21-loaded TDEVs using (liver cells) Hepg2 and SKB3 (for BC) cell lines, whereby significant homologous and heterologous transfection along with intracellular Cy5anti-miR delivery. Monitoring the drug transport activity of TDEV-loaded carriers in BC cells, the investigators noticed that a TDEV-driven anti-miR-21 delivery decreased the doxorubicin resistance in BC cells, alongside triplicated cell death efficacy compared to an unaided doxorubicin delivery. Encouraged by these attributes of TVs, the investigators tried to use them as functionalization agents for the Au-coated iron oxide NPs (Au-coated FeO NPs). The TDEV functionalized Au coated FeO NPs exhibited splendid T2 contrast during the in vitro conducted MRI along with a significant photothermal effect in 4T1 cells. It was noticed that TDEVs functioned well in combinations with Au-coated FeO NPs as also demonstrated by *tumor targeting and intracellular localization* ability of TDEV-GIONs using MRI. So this study established the compatibility of TDEV for enhanced tumor cell localization activity in combination with Au-coated FeO NPs. The results could be propagated further for the use of TDEVs from other organs and the combination of several other NPs for a sustained drug delivery ensuring minimized intracellular resistance (Bose et al. 2018).
- Moving to 2019, the first significant study used a nanoconjugate (NCg) (consisting of curcumin-loaded Au-PVP (polyvinylpyrrolidone) NPs) conjugated with folic acid. Following preparation, the NCg was characterized by UV-Vis, FT-IR spectroscopy, X-ray powder diffraction, and thermogravimetric analysis. The in vitro anticancer and antimigratory attributes of NCg were studied using

MTT and wound migration assays while the in vivo anticancer ability was studied in a preclinical BC orthotopic mouse model. The NCgs size and charge played a key role in curcumin release, analyzed using layer-by-layer assembly. Nearly 80% curcumin was released in an acidic pH and the as-prepared NCg did not exhibit any aggregation on being incubated with human serum besides being *mimicked with intrinsic peroxidase* behavior in the presence of 3,3′,5,5′-tetramethylbenzidine substrate. Results of anticancer studies of these NCgs using the MTT assay revealed an enhanced activity corresponding to lower doses in estrogen/progesterone receptor-negative cells compared to ER/PR positive cells. Along with this, in vivo inspection of prepared NCgs inhibited the cell migration with high antitumor ability. The efficacy was reported specifically on tumor cells and no cytotoxicity was observed corresponding to the tested concentrations in normal human breast epithelial and mouse fibroblast cells. Thereby, *PVP and folic acid* aided in the tumor cell-specific activity of curcumin which itself is well-known for its selective actions toward tumor cells. The observations create interest in using PVP as a support matrix for the delivery of many other polyphenols like curcumin (Mahalunkar et al. 2019).

- The subsequent 2019 attempt screened the in vitro chemotherapeutic efficacy of ZnO NPs delivered via mesoporous silica nanolayer (MSN) to the drug-sensitive (MCF-7, ER-positive, CAL51, triple negative) and drug-resistant (MCF-7TX, CALDOX) BC cells. The ZnO NPs coated on MSNs were coated on Au nanostars (shape diversity of Au-NPs) for testing the NIR-II ranged imaging capabilities. Inspection using *electron and confocal microscopy* inferred an accumulation of MSN-ZnO-Au nanostars close to the plasma membrane and a *parallel internalization* by the BC cells. High-resolution microscopy analysis of the MSN-loaded ZnO NPs inferred a degradation of MSN coating outside the cells, releasing the ZnO NPs for interaction with plasma membranes. Together, the ZnO NPs loaded over the MSN reduced the viability of all tumor cell lines, of which the CAL51/CALDOX cells were more susceptible. Monitoring the targeted activity, the as-prepared MSN-loaded ZnO NPs were conjugated with Ab to Frizzled-7 (overexpressed receptor in several BC cells). This conjugation was made using disulfide linkage (-S-S-) which is easily cleaved by a high glutathione concentration inside the tumor cells. As a result of the cleavage, the Zn^{+2} were released into the cytoplasm. It was noticed that the *Frizzled-7 targeting enhanced the MSN-ZnO-AuNs toxicity* by more than three times, toward the drug-resistant MCF-7 cell line (which exhibited the highest Frizzled-7 expression). Overall, the study illustrated MSN-loaded ZnO NPs as promising anticancer agents to treat triple-negative and drug-resistant BCs besides highlighting a target-specific ability of Frizzled-7 (Ruenraroengsak et al. 2019).
- The year 2020 continued the interest in Au-NPs aided drug delivery to BC cells, with the first attempt involving the use of the Turkevich method prepared Au-NPs for a combinatorial delivery of doxorubicin and polo-like kinase (PLK1, a siRNA) to the SKBR3 cell line. The Au-NPs were coated with polyethylenimine (PEI) which aided in PLK1 surface assembly. Doxorubicin was loaded on NPs via a pH-sensitive linker, having a thiol group at one terminal, which aided in

sustained drug release. Therapeutic activity of doxorubicin-PLK1 combine was assessed in 2D and 3D cultured systems, where reduced IC_{50} extent (0.06 ± 0.002) showed a *synergistic response of doxorubicin and PLK1* (siRNA) gene delivery. The response was compared with unaided doxorubicin (0.805 ± 0.29), individual doxorubicin, and PLOK1 delivery with Au-NPs (0.122 ± 0.02 and 0.512 ± 0.3, respectively). The PEI-stabilized Au-NPs exhibited a core-shell morphology with *Au-NPs forming the core and PEI conjugated to the surface through a PEG linker* (anticipated as biocompatible and nontoxic). Thiol bonds served as stability augmenting factors in the combined delivery which conjugated the doxorubicin simultaneously with Au-NPs. The optimized vehicle configuration also offered a provision to conjugate other drugs to the Au-NP surface for developing a multidrug delivery response. The inclusion of Au-NPs as core in the vehicle always provides an option to incorporate photothermal therapy besides the imaging component for higher localized toxicity and improved diagnosis of the tumor progress (Shrestha et al. 2020).

- Subsequent 2020 comprises a slightly different than conventional attempt, using green nanotechnology as an initiative, whereby *mango peel constituent phytochemicals* were used as reducing agents to form Au-NPs. Briefly, 30 mg of dry mango peel powder was added to 6 ml of double distilled water in a 20 ml vial, followed by 10 min of stirring at RT till a homogenous suspension is obtained. Thereafter, 100 µl, 0.1 M $NaAuCl_4$ solution was added following which its color changed to ruby-red within 2 min. Although polyphenols are frequently recommended and used as reducing agents for NPs capping activities the mango peel phytochemicals (the major ingredient is Mangiferin) create an interest in the natural essence of the study. Retrieved Au-NPs were termed as *Nano Swarna Bhasma* (NSB drug), which was tested for (i) anticancer attributes in breast tumor cells, (ii) preclinical therapeutic efficacy studies in BC bearing severe combined immunodeficiency syndrome (SCID) infected mice via oral delivery, and (iii) first-ever analysis of clinical translation from mice to human BC patients (using pilot human clinical trials. The study comprised a part of *Ayurveda, Yoga and Naturopathy, Unani, Sidha, and Homeopathy* (AYUSH) initiative by the Government of India. Preclinical in vitro (MDA-MB-231 BC cell line) and in vivo investigations in breast tumor-bearing mice unanimously established the NSB (the Ayurvedically fabricated Au-NPs) as being highly effective in controlling the BC growth, exhibiting a dose-dependent response. This was the reason to pursue clinical trials in humans, wherein, patients treated with NSB drug capsules along the standard of care treatment (Arm B) expressed 100% clinical benefits compared to patients administered treatment with Arms A. This indicated significant clinical benefits of NSB drugs in adjuvant therapy which could improve the therapeutic response of multiple chemotherapeutic drugs (Khoobchandani et al. 2020).
- Another 2020 milestone attempt using Au-NPs as a drug delivery carrier for BC treatment used Au-NPs to facilitate a significant uptake of hesperidin, a *flavonoid glycoside* being listed for its manifold therapeutic activities. The major hurdles in the action of this flavonoid include it's below par AQ solubility, which inhibits its

trafficking to the desired tumor site. The study from Iraq and Saudi Arabia's joint efforts involved a chemical synthesis-driven hesperidin loading on the Au-NPs. Manifold characterizations, including UV-Vis spectroscopy, FT-IR, XRD, FESEM, TEM, EDX, and ζ-potential measurements, confirmed the chemical synthesis of hesperidin conjugated Au-NPs (Hsp-Au-NPs). The cytotoxic effect of Hsp-Au-NPs on human BC cell line (MDA-MB-231) was ascertained using MTT and crystal violet assays. Analysis revealed a considerable decrease in the cell population along with an inhibition in the growth of treated cells when compared to normal human breast epithelial cell lines (HBL-100). Determination of apoptosis was made using a fluorescence microscope, using acridine orange propidium iodide dual staining assay. The in vivo study was conducted in mice to ascertain the Hsp-Au-NPs toxicity, and to ascertain which, expressions of *hepatic and renal functionality markers* were assessed. No significant distinctions could be found for the tested indicators, complemented by null apparent damages shown in the histological images of liver, spleen, lung, and kidney, after Hsp-Au-NPs treatment. The Hsp-loaded Au-NPs were found to ameliorate the functional activity of macrophages against Ehrlich ascites tumor cells bearing mice, complemented by the *inhibited IL-1β, IL-6, and TNF secretions* post-Hsp-Au-NPs treatment (Sulaiman et al. 2020). Thus, this study comprised another attempt toward explaining the significance of Au-NPs as efficient carriers of natural polyphenols, which improved their structural expression along with enhanced specificity.

- Toward the end of 2020, another study similar to the previous attempt, reported the improved anticancer potential of cisplatin (CDDP), through its Au-NPs-mediated delivery to human breast cancer (SKBR3) and normal (MCF-10A) cells. The attempt used L-lysine as a linker to conjugate cisplatin with Au-NPs. The as-developed nanodrug (Au-NPs-Lys-CDDP) was rigorously characterized using UV-Vis spectroscopy, electron force microscopy, particle size, and ζ-potential measurements. Apoptosis and morphology variations were screened using Flow Cytometry and acridine orange/ethidium bromide (AO/EtBr) staining respectively. The CDDP-loaded Au-NPs through lysine revealed 85 nm as particle size alongside a -25 mV ζ-potential, inferring an efficient uptake by tumor cells. The CDDP-loaded Au-NPs were distinguished from Au-NPs by a strong absorption shift in the 525 nm region, besides eight-*fold lower* IC_{50}, against the SKBR3 cell lines. The CDDP delivery on conjugation with Au-NPs caused a substantial enhancement in the SKBR3 apoptosis, with *minimal cytotoxic effects on normal cells*. So, this study is yet another piece of evidence to prove that Au-NPs could efficiently function as sustained carriers of chemotherapeutic drugs, with reduced dosage, a sustained release profile, and a localized toxicity expression (Ganji et al. 2020).

5.5 Silver Nanoparticles for Breast Cancer Treatment

- Unlike Au, Ag NPs use BC treatment more focused on the utilization of a higher Ag^{+} intracellular toxicity. It is because of this reason that contrary to most Au-NPs studies using Au-NPs as drug carriers, those with Ag NPs either use them in the native form as tumor treatment agents or just to enhance the cytotoxicity of a moderately toxic drug such as several polyphenols. Therefore the exact intent of Au and Ag NPs treated BC is distinguished via the carrier abilities of the former and the drug-like functioning of Ag NPs.

  A cautious glance at the BC pathophysiology infers considerable changes in the receptor expression patterns on the cell surface, together resulting in tumor progression and metastasis. The most common ligands used to deliver chemotherapeutic drugs through nanocarriers are (i) albumin and (ii) trastuzumab. Albumin is a protein well known for its caveolae-mediated endocytosis, whereas Trastuzumab is a humanized MAb, with a HER2 receptor binding specificity. Besides these two ligands, the most relied upon ligands used in nanocarrier-mediated drug delivery to BC are agglutinins and glycoproteins, both specifically binding to sugars. These findings suggest that carbohydrates are the most suited ligands for nanocarrier-mediated chemotherapeutic drug delivery to cancer cells, with significant regulatory influence on cell-cell and cell-ECM interactions.
- Beginning from select 2019 studies, the first from the United States was inspired by the ability of Ag NPs to treat aggressive cancers, based on which the investigators attempted to correlate the physicochemical attributes of Ag NPs with their biological activities. As a result, the study involved a thorough structure–function analysis, mechanistic, safety and efficacy evaluation *vis-à-vis* Ag NPs ability to treat TNBCs. Of note, TNBCs are the most dreaded tumors in terms of their mortality contribution among the BC deaths. The findings revealed cytotoxicity of Ag NPs to TNBC cells which were mediated independent of their sizes and shapes and used stabilizing agents. However, this toxicity is selective for tumor cells and toward benign breast epithelial cells, no significant toxicity was noticed. Analysis of cytotoxicity expressions in TNBCs and nonmalignant breast epithelial cells revealed their similar sensitivity, inferring a necessity for nanoparticle formulation for a TNBC-specific toxicity induction. It was observed that despite being internalized by TNBC and nonmalignant BC cells, the Ag NPs undergo swift degradation only within TNBC cells. The distinct response of Ag NPs within the TNBC cells is due to the depleted intracellular antioxidants (such as glutathione) that cause endoplasmic reticulum (ER) stress. Such degradation is however not observed in nonmalignant breast epithelial cells. Separate studies revealed extensive DNA damage in 3D TNBC tumor nodules (in an in vitro setting) on being exposed to Ag NPs but no alterations were observed in the normal architecture of breast acini in 3D cell culture, nor any sort of DNA damage or apoptotic induction. The study also revealed the effectiveness of systematically administered Ag NPs at nontoxic dosages toward a reduced growth of TNBC *tumor xenografts in mice*. Thereby, on the whole, this study elucidates the TNBC treatment efficacy of Ag NPs as safe and specific moieties (Swanner et al. 2019).

- Another significant 2019 attempt matched the yester attempt in terms of ER stress interception of Ag NPs within the BC cells. Citing the repeated development of multidrug resistance (MDR) in BC cells, the study from Hungary observed size-dependent cellular activities of Ag NPs toward modulated P-glycoprotein interception and the counteracting of MDR in the BC cells. Evaluating the antitumor efficacy of 5 and 75 nm Ag NPs in the TNBC cells, the investigators noted that 75 nm Ag NPs were more pronounced in decreasing the P-glycoprotein (Pgp) efflux activity which reduced the resistant response toward doxorubicin. Further, it was observed that these activities of 75 nm Ag NPs were due to depleted ER calcium levels which *resulted in ER stress and decreased plasma membrane Pgp positioning*. Thereby, this study provided a mechanism to reduce the MDR through enhanced ER stress, which can be extended to other chemotherapeutic drugs too (Gopisetty et al. 2019).
- The year 2020 further witnessed intensified attempts to use Ag NPs for BC treatment, wherein Ag NPs individually and with other chemotherapeutic agents, have induced sustained toxicity to moderate or eliminate tumorigenic activities. The first of these attempts comprised a collaborative attempt from Saudi Arabia and Egypt, which examined the effect of silver citrate NPs (Ag NPs-CIT) on NF-κB activation alongside tumor necrosis factor (TNFα) mRNA/protein expressions in the phorbol myristate acetate (PMA)-stimulated MCF-7 human BC cell line. It is pertinent to mention here that NF-κB is the prominent transcription factor responsible for the activation of several inflammatory mediators (including TNFα), having a lethal association with tumor onset. The Ag NPs used herein were prepared using the citrate reduction method and characterized for spherical morphology having (19.2 ± 0.1 to 277 ± 0.12) nm particle sizes and(-9.99 ± 0.8 to -34.63 ± 0.1) mV surface charges. The as-prepared Ag NPs exhibited a UV absorption wavelength within 381 to 452 nm range, produced >40% DPPH radical scavenging, and were nontoxic to MCF-7 BC cells besides inhibiting PMA-induced NF-κB, p65 activation along with TNFα mRNA/protein expressions. Analysis revealed significantly inhibited TNFα expression upon being administered with Ag NPs-CIT through *suppressed NF-κB signaling* in simulated BC cells. The study, therefore, explained the anticancer and anti-inflammatory attributes of silver citrate NPs. The results could be extended to other NPs through modulated TNFα activities and it would be a paradigm shift if Ag NPs prepared using plants, micro-organisms, and physical methods (such as electrodeposition, laser ablation) are compared for their TNFα-driven anticancer response for tumors other than those of breast (Abdellatif et al. 2020).
- The second rigorous attempt is a study from China wherein the nonspecific and below-par tumor sensitization ability of Au-NPs (due to tumor radio-resistance and nontarget tissue irradiation) was improved using hyaluronic acid modified Au-Ag alloy NPs (Au-Ag@HA NPs). The Au-Ag@HA NPs were prepared through $AgNO_3$ and $HAuCl_4$ coreduction (using trisodium citrate as a reducing agent) followed by HA surface modification. The intent of conferring HA modification to Ag-Au alloy NPs was to improve *their preferential targeting to 4T1 BC cells* that over-express the CD44 receptor. The introduction of Ag in the

Au-NPs imparted superior multienzyme-like activities for efficient tumor catalytic therapy. Monitoring the antitumor activity of Au-Ag@HA NPs, investigators noted the ionizing radiation and peroxidase-like activity which together boosted the generation of •OH radicals along with toxic Ag^{+} release at the tumor sites. Overall the results summarized a mechanism for improved nanozyme and Ag^{+} combined therapy against cancer and could advance the development of *multifunctional nanoplatforms for synergistic antitumor* activities. The role of stoichiometry optimization is important it keeps the monodispersed state of NPs intact (Chong et al. 2020).

- Yet another significant 2020 attempt is a study from the collaborative attempts of Egypt and Saudi Arabia, wherein cefotaxime (a drug)-conjugated Ag NPs (Cf-Ag NPs) were synthesized to counter the MDR bacterial infections. The study analyzed the cell death status of Cs-Ag NPs along with the apoptotic pathways of human RPE-1 normal and MCF-7 BC cells. The exact purpose was to screen the antimicrobial efficacy of cefotaxime conjugated Ag NPs against the cefotaxime-resistant *E. coli* and MRSA strains. Morphology analysis of the Ag NPs in neat state and cefotaxime conjugated states were 7.42–18.3 and 8.48–25.3 nm with a spherical shape. Compared to neat Ag NPs and pure antibiotics, the Cf-Ag NPs were strongly antimicrobial, with a MIC ranging between 3 and 8 $\mu g \bullet ml^{-1}$ against *E. coli* and *MRSA* bacterial stains. Apart from this, the Cf-Ag NPs exhibited no cytotoxic effect on normal cells even at 12 $\mu g \bullet ml^{-1}$ till 24 h. The IC_{50} for the Ag NPs in a neat state and of Cf-Ag NPs was computed as 12 $\mu g \bullet ml^{-1}$ toward human RPE-1 normal and human MCF-7 BC cell lines. To counter the inferences of apoptosis-mediated cell death, monitoring of proapoptotic genes (p53, p21, and Bax) and antiapoptotic genes (Bcl-2) was done, which revealed upregulated and suppressed activities. These distinctions were noticed on 48-h treatment of NPs at 24 $\mu g \bullet ml^{-1}$ dosages, which was subsequently chosen as the most effective concentration. Thus, this study reported a mechanism using Ag NPs, to revive the anticancer activity of old and unresponsive cefotaxime, which were subsequently expressed as cefotaxime-CS-Ag NPs (Halawani et al. 2020). Similar trials should be extended for other metallic NPs and also for their possible combinations with individually nonsuited chemotherapeutic drugs.
- Another milestone attempt is a study from Turkey, wherein anticancer activities of *Cuminum cyminum L.* (Cumin) seed extract, chemically Cumin seeds) and biologically (using synthesized Ag NPs were evaluated on human breast adenocarcinoma cell line (MCF-7) and human breast adenocarcinoma metastatic cell line (AU565). The as-synthesized NPs were thoroughly characterized for their nanoscale attributes using familiar spectroscopic techniques, dispersion profile assessment (using DLS), and morphology assessment using SEM. Cytotoxic and anticancer activities of chemically and biologically prepared Ag NPs were screened using an MTT assay. It was noticed that biologically prepared Ag NPs ($IC_{50} = 1.25\ \mu g \bullet ml^{-1}$) were less toxic against *J774 macrophage cells* compared to chemically prepared NPs ($IC_{50} = 0.75\ \mu g \bullet ml^{-1}$). The biologically prepared Ag NPs also caused significant inhibition of human BC cells at nontoxic

concentrations of 0.25 and 0.5 μg•ml^{-1}. However, at elevated concentrations, the lethal effects of chemically prepared Ag NPs on BC cells were significantly higher than biologically prepared Ag NPs. Monitoring the antitumor activities of cumin extract, it was found that it killed half of the MCF-7 cells but did not show any inhibitory effect on AU565 cells. Contrary to this, chemically prepared Ag NPs caused 95% and 97% inhibitions in the MCF-7 and AU565 cell lines corresponding to the highest dosage, 12.5 μg•ml^{-1}. The similar extents for biologically prepared Ag NPs were estimated as 87.5% and 96%. Since the biologically prepared Ag NPs did not exhibit any residual toxicity (in their native state), these could be pioneer agents as anticancer drugs for a future BC treatment. Similar prospects could be extended to cumin similar plant products, having a matching composition of their respective extracts (Dinparvar et al. 2020).

- In a subsequent elegant attempt, Gonclaves and colleagues from China studied the toxicity and cell internalization of individual (Ag and Au), bimetallic (Ag-Fe, Au-Fe, and Ag-Au) aminolevulinic acid (ALA) NPs. Of note, ALA can regulate the generation of protoporphyrin IX, an intracellular photosensitizer commonly used in photodynamic therapy. The NPs were prepared using the photoreduction method and characterized thoroughly using spectroscopic, dispersion, and morphology. The amount of singlet oxygen generated by a yellow LED and ultrasound was studied for Au, Au-Fe, and Ag-Au-NPs. To examine the characteristic impact on MCF-7 cells, the cytotoxicity assays of MCF-7 were performed in presence of NPs besides a quantification of PpIX fluorescence using high content screening (HCS). Inspection revealed the development of red fluorescence after 24 h of NPs incubation with MCF-7 cells, suggesting an efficient conversion of ALA on the NPs surface to PpIX. Best results for singlet oxygen generation with LED or ultrasound generation were retrieved for ALA: Ag-Au-NPs. So, this study illustrated the significance of efficient ALA delivery to BC cells besides the *generation of singlet oxygen and ALA to PpIx conversion* inside the cells, which paved way for *photodynamic and sonodynamic therapies*. The results could be amicably extended to the emerging studies on simultaneous NPs and photoradiation administration to cancer cells, whereby improved and sustained toxicities could be accomplished (Gonclaves et al. 2020).
- In a highly significant attempt this year, Ulagesan and colleagues studied the cytotoxicity and anticancer activities of Ag NPs, prepared using aqueous extract of marine alga *Capsosiphon fulvescens* against the MCF-7 BC cells. The as-formed Cf-Ag NPs were thoroughly characterized for their nanoscale attributes, revealing a 20- to 22-nm particle size, spherical morphology, and null aggregation. Analysis for cytotoxicity revealed an IC_{50} of 50 μg•ml^{-1}, whereby the viability of treated MCF-7 cells decreased on increasing Cf-Ag NPs concentrations. Morphology evaluation of the MCF-7 cells before and after treatment with Cf-Ag NPs revealed irregular confluent aggregates with round polygonal cells on being treated with the aqueous algal extract (devoid of Cf-Ag NPs). Contrary to this, the Tamoxifen and Cf-Ag NPs treatment resulted in significant morphological alterations. Analysis of apoptosis using 4′,6-diamidino-2-phenylindole (DAPI) staining, wherein Cf-Ag NPs treated MCF-7 cells developed a bright blue fluorescence with an evident,

shortened, and disjointed chromatin. The control cells, on the contrary, developed less bright fluorescence. Analysis using Flow Cytometry revealed a high percentage of cells in the late apoptosis, on being treated with Tamoxifen (77.2%) and Cf-Ag NPs (74.6%). So this study established another worthwhile analysis method to use Cf-Ag NPs as BC treating agents with a sustained cytotoxicity profile. An element of concern, however, is the in vitro conduct of the study which provides a caution to validate the results in the in vivo conditions using the same concentrations of Cf-Ag NPs. It is well known that nearly 90% of the in vitro results are not exactly replicated when examined in vivo conditions (Ulagesan et al. 2021).

The above discussion amply illustrates the therapeutic significance of Au and Ag NPs *vis-à-vis* drug delivery to respective tumor sites in the lungs and breast. The success of NPs use as drug carriers is highly dependent on their targeted engineering for which one needs to have a thorough knowledge of the stage of the cancer cells being treated. This is because the intensity of cell surface receptors is not the same during different tumor stages and a distinct expression of these may result in totally different drug internalization extents within the tumor cells. Even though drug delivery using NPs is in practice for more than two decades (although its commercialization gained momentum in the last decade) and there is plentiful literature explaining the delivery modes along with corresponding pros and cons, still there are multiple myths that hinder a clear understanding of this subject. As also highlighted in some of the discussed studies, the efficacy of NPs-mediated drug delivery does not depend on the implicit particle sizes and larger-sized particles may be internalized more efficiently within the tumor cells. There are many reports of nanorods being more effective in localized toxicity induction within the tumor cells. So, the concern does not focus solely on particle size in fact, it is the binding of the drug with the NPs which is an important regulating factor. The delivery process mandates that once the drug-loaded NPs are within the tumor cells, *they should be capable of releasing the bound drug spontaneously* due to the physicochemical dynamics of tumor cell activities. Alternatively, it necessitates that the body's energy must not be expended for releasing the drug at the needed site. Secondly, the drug-loaded carrier must be capable of the requisite extent of opsonization, so that the detection of the drug as a foreign entity does not metabolize it out before it reaches its inside the tumor cell. Therefore, *surface engineering concerning stealth delivery* must be properly understood so that the maximum amount of drug reaches the tumor cell. A third and most important factor is the dosage moderation extent which implies that once we are using engineered NPs as delivery vehicles, a reduced amount of drug loading should be attempted contrary to the unaided drug delivery. Thereby, the acronym, *ADME* (i.e., *Adsorption, Degradation, Metabolism, and Excretion*) must be followed with the highest caution to achieve success in the NPs aided drug delivery. The net conclusion is that the *binding of the drug with the carrier must not be through drastic forces* which could damage the native drug chemical structure and the task of prolonging the intracellular stay of a drug must be accomplished. The latter part

implies the prevention of prompt chemical degradation from the physiological environment which is prevented by the stealth delivery using NPs.

6 Possible Cautions for Unpredictable Nanoparticle Toxicity

- Although much has been accomplished in terms of enhanced specificity and dosage moderation (ensuring minimal side effects), the smaller size conferred gravitational freedom along with engineered surfaces, creating several vulnerable prospects in terms of the ultimate fate of the NPs, once these are inside the body. The *undesirable physiologically deleterious* activities of Au and Ag NPs are the implicit functions of their physicochemical nature, their engineered surfaces, and unconventional chemical properties that are the major causes of the enhanced interaction potential. The foremost threat of toxic behavior is its *unpredictability due to which no database or records or earlier studies* have reduced the vulnerable proportions in the relatively recent attempts. The sole cautionary mechanism has been to reproduce the trials on an in vitro scale with an ensuring of stealth trafficking.
- The lungs, liver, kidneys, and spleen are the major organs where the administered NPs gat deposited after their intended applications of delivering the drug(s) are completed. Tables 4, 5, and 6 provide a glimpse of select studies wherein Au and Ag NPs retention in these organs was noticed on being delivered to rats and mice. The variable *damage extents and residence times* are the implicit functions of characteristic particle sizes, engineered surface coatings, and preparation sources. In several studies and noted review articles, the toxicity of NPs has been reported as random and majorly mediated via distinctive capping agents. A 2017 study aimed at investigating the *biodistribution and toxicological* impacts of Ag and Au-NPs through their repetitive intravenous administration in mice revealed Au-NPs to be primarily deposited in the liver while the Ag NPs were preferentially accumulated in larger organs, comprising the heart, lungs and kidneys. The circulation of NPs in blood and fecal excretions showed higher contents of Ag NPs. The analysis of mouse body and organ mass, hematology, biochemistry, and histopathology revealed a slight toxicity difference between Ag and Au-NPs over two months duration. The analysis of RT-qPCR showed Ag NPs to induce more pronounced gene expression changes which culminated in enhanced oxidative stress, apoptosis, and ion transport (Yang et al. 2017).
- Another noted and very recent contribution by Ferdous and Nemmar rigorously discusses the various factors for enhanced toxicity of Ag NPs on an in vivo scale. Holding PCPs as major culprits behind the adverse reaction initiation, the authors have exclusively discussed the particle sizes and varying nature of engineered surface coatings toward the distribution and kinetics of Ag NPs. For instance, a small particle size attributes to a high specific surface area due to which a greater proportion of atoms are displayed on the surface. This infers that for the same mass of two or more NPs, *biological interactions and toxicity are more dependent on particle number and SA than particle mass*. The source further mentions that

Table 4 Select studies of Au-NPs administered to rats and mice, along with their tissue accumulation and dosage-dependent toxicity induction

Size and dose of examined Au-NPs	The animal model studied and exposure duration	Tissue accumulation	Conclusive findings
20 nm, 0.01 mg·kg^{-1}	Rats, 1–7 days, 1–2 months	Liver, spleen, lungs, kidneys, heart, urine, and feces (>25 g)	Wide deposition over 2 months, single exposure resulted in manifold gene expression changes
1.4, 5, 18, 80 and 200 nm, 1.6 ± 0.2 to 43.7 ± 5.3 μg per rat	Rats, 1 day	Liver, spleen, kidney, lungs, heart, brain, uterus, blood, gastrointestinal tract	Size and surface charge-dependent diffusion, NPs deposited in the liver increased from 50% of 1.4 nm to >99% 200 nm
PEGylated Au Au-NPs of 11, 21 and 31 nm, 0.07 to 0.30 mg·kg^{-1}	Rats, 1 h, 1 day	Liver, spleen, blood, Gastrointestinal Tract, lungs, kidney, heart, urine, brain, and feces	10 kDa PEG-modified NPs exhibited prolonged blood circulation
10, 50, 100 and 250 nm; (0.077 to 0.108) mg·kg^{-1}	Rats, 1 day	Liver, spleen, blood, lungs, kidney, heart, thymus, brain, and testes	Size-dependent tissue distribution with 10 nm particles showing a widespread organ distribution
15, 50, 100 and 200 nm; 1000 mg·kg^{-1}	Mice, 1 day	Liver, lungs, kidney, spleen, heart, brain, blood, stomach, and pancreas	Size-dependent tissue distribution with 15 and 50 nm particles crossing the BBB
13 ± 1 nm, 0.004–0.04 mg·kg^{-1}	Rats, 1 h, 1 day	Liver, spleen, blood, kidney, Gastro-Intestinal Tract, feces, lungs, uterus, heart, urine, and brain	In vivo proteolytic degradation of polymer coated Au Au-NPs having a high colloidal stability
16.1, 0.7 mg·kg^{-1}	Rats, 0.5 h, 28 days	Liver, spleen, heart, lungs, blood, bones, thymus, kidneys, brain, muscle	Surface coating affected toxicity more than the biodistribution
1.4, 18 nm; 0.011 and 0.11 mg·kg^{-1}	Rats, 1 day	Liver, urine, kidneys, feces, blood, Gastro-Intestinal Tract, spleen, uterus, heart, and brain	Accumulation of 18 nm particles in the spleen and liver is greater than 1.4 nm particles
6 nm, 11.4–13.3 mg·kg^{-1}	Mice, 28, 56 days	Liver, spleen, kidneys, heart, lungs, feces, blood, brain, stomach, and intestines	Particles were majorly deposited in the liver,

the various coatings intended to enhance the functionalization of Ag NPs change their fate and toxicity. The familiar coatings applied to Ag NPs to improve their biocompatibility and stability *vis-à-vis* random aggregations include the materials

Table 5 Effects and distribution of Ag NPs following inhalation-driven pulmonary exposure in rodents

Particle size and animal model	Dosage	End-point measurement	Effect & tissue accumulation
11–14 nm, Sprague-Dawley rats	1.73×10^4 per cm^3 (low dose), 1.27×10^5 per cm^3 (middle dose), 1.32×10^6 per cm^3 (high dose)	6 h per day, 5 days per week for 4 weeks, sacrificed 1 day after 1 day of last exposure	Though NPs were accumulated inside the lungs (Lu), liver (Li), brain (Br), and olfactory bulb (Ob) but no significant toxic outcomes were noted
18 nm, Sprague-Dawley rats	0.7×10^6 particles per cm^3 (low dose), 1.4×10^6 particles per cm^3 (middle dose), 3×10^6 particles per cm^3 (high dose)	6 h per day, 5 days per week for 90 days, sacrificed 1 day after 1 day of last exposure	Subchronic exposure compromised the lung function but no tissue accumulation was noticed
18 nm, Sprague-Dawley rats	0.6×10^6 particles per cm^3 (low dose), 1.4×10^6 particles per cm^3 (middle dose), 3×10^6 particles per cm^3 (high dose)	6 h per day, 5 days per week for 4 weeks, sacrificed 1 day after 1 day of last exposure	Gender-dependent kidney accumulation, dose-dependent increase of bile duct hyperplasia in Ag NPs exposed liver, accumulation in lungs, liver, olfactory bulb, kidney, and brain
10 nm (PVP coated), Male C57BI/6 mice	(3.3 ± 0.5) mg·m^{-3}	4 h per day, 5 days a week for 10 days, sacrifice at 1 h and 21 days since the last exposure	Subacute inhalation induced minimal pulmonary toxicity, and lung accumulation was noticed
15 nm, 410 nm, Male Fischer rats	179 μg·m^{-3}, 167 μg·m^{-3} for 15 and 410 nm respectively	6 h per day, 4 consecutive days, sacrifice at 1 and 7 days postexposure	Size-dependent effect on pulmonary toxicity on inhalation of similar mass concentration of both sizes, accumulation in lungs and liver
15 nm, Brown Norway and Sprague-Dawley rats	8, 28 μg	3 h per day and 3 h per 4 days, sacrifice on one and 7 days postexposure	Acute pulmonary neutrophilic inflammation with the generation of proinflammatory and proneutrophilic cytokines, accumulation in lungs
20 nm, 110 nm, Male Sprague-Dawley rats (CT coated)	7.2 ± 0.8 mg·m^{-3} and 5.3 ± 1.0 mg·m^{-3} for C20 and C110, respectively	Six hours per day, sacrifice at 1, 7, 21, and 56 days postexposure	Delayed peak and short-lived inflammatory and cytotoxic effects in lungs, greater response due to smaller sizes

(continued)

Table 5 (continued)

Particle size and animal model	Dosage	End-point measurement	Effect & tissue accumulation
18–20 nm, Female C57BL/6 mice	3.80×10^7 particles per cm^3	One hour per day for the first 15 days of gestation, sacrificed 4 h after exposure	Increased number of resorbed fetuses associated with reduced plasma estrogen levels on 4-h per day exposures, lung, spleen, liver, and placental accumulation

such as trisodium citrate (CT), and cetyltrimethylammonium bromide, polyvinylpyrrolidone, poly(L-lysine), bovine serum albumin, Brij 35 and Tween-20. Of these, CT and PVP are more commonly used as aggregation stabilizers. The coating on the NPs surface modifies their toxic effect in cells, as the NPs which are positively charged gain spontaneous entry inside the cells due to a negative surface polarity of the plasma membrane. However, the positively charged NPs are randomly sensitive and get involved in harmful side reactions, resulting in accumulated free radicals. Their random interactions make it tougher to excrete them, owing to which these inevitably manifest as toxic intermediates. The different kinds of surface corona generated from the *interfacial interactions of Ag NPs and biological fluids* result in composition-dependent alterations in Ag NPs dissociation as Ag^+ and ultimately the toxicities (Ferdous and Nemmar 2020).

- The toxicity of Ag NPs is substantially due to the Ag^+ formation, which happens at a faster rate for the smaller Ag NPs (a larger SA). A faster Ag^+ generation leads to its rapid dissolution in the surrounding microenvironment which further leads to *increased bioavailability, enhanced distribution, and higher toxicity.* The release rate of Ag^+ depends on several factors including size, shape, concentration, capping agent, and the specific colloidal state of NPs. The released Ag^+ spontaneously interacts with respiratory chain proteins on the membrane, interfering with *the intracellular O_2 reduction and inducing a rapid ROS generation*, ultimately resulting in elevated oxidative stress. Major access modes of NPs comprise *ingestion, inhalation, skin contact, and direct systemic circulation* via intraperitoneal or intravenous injection.
- Figure 19 (above) depicts the different exposure modes to Ag NPs, illustrating their biodistribution and the corresponding mechanisms underlying the corresponding effects. The major toxicity events initiated by the exposure of Ag NPs include aggravated inflammation and oxidative stress. The Ag NPs due to their smaller dimensions easily invade the different biological barriers and gain entry within the systemic circulation. This biological and cellular invasion happens more readily for the intravenously administered NPs. Subsequently, the Ag NPs get manifested in different organs and result in organ-specific

Table 6 Effects and distribution of Ag NPs following pulmonary exposure in rodents on intratracheal instillation

Particle size and animal model	Dosage	End-point measurement	Effect & tissue accumulation
20 nm and 110 nm (PVP and CT coated), Male-Sprague-Dawley rats	0.5, 1 $mg \cdot kg^{-1}$	Single intratracheal instillation, sacrifice at 1,7 and 21 days postexposure	Coated and size-dependent retention in lungs, PVP coated particles had less retention over time and larger NPs were cleared earlier from large airways, lung accumulation noticed
20 nm and 110 nm (PVP and CT coated), Male-Sprague-Dawley rats	0.1, 0.5, 1 $mg \cdot kg^{-1}$	Single intratracheal instillation, sacrifice at 1,7 and 21 days postexposure	Smaller-sized NPs caused more inflammatory and cytotoxic responses, larger particles caused lasting effects to post 21 days of installation, and no tissue accumulation was noticed
200 nm CT capped NPs, Male-Sprague-Dawley rats	1 $mg \cdot kg^{-1}$	Single intratracheal instillation, sacrifice on 1 and 7 days postexposure	Exacerbation of cardiac ischemic-reperfusion injury, no accumulation noticed
20 nm, 110 nm, Male-Sprague-Dawley rats	1 $mg \cdot kg^{-1}$	Single intratracheal installation on 1 and 7 days postexposure	Exacerbated cardiac injury one day after installation, irrespective of a capping agent. Injury lasted longer for 110 nm PVP capped Ag NPs following 7 days installation, no accumulation noticed
50 nm, 200 nm (PVP coated), Female Wistar rats	0.1875, 0.375, 0.75, 1.5, 3 $mg \cdot kg^{-1}$	Single intratracheal instillation, sacrifice on 3 and 21 days postexposure	Focal deposition of Ag in peripheral organs along with transient lung inflammation, liver, spleen, and kidney deposition
50 nm, 200 nm (PVP and CT coated), Female BALB/C mice	0.05, 0.5 and 2.5 $mg \cdot kg^{-1}$	Single intratracheal instillation, sacrifice one-day post instillation	Size, dose, and coating-dependent proinflammatory effects in healthy and sensitized lungs, no accumulation noticed
10 nm, BALB/C mice	0.05, 0.5, 5 $mg \cdot kg^{-1}$	Single intratracheal instillation, sacrifice at 1 and 7 days postexposure	Oxidative stress, DNA damage, apoptosis in the heart, induced prothrombotic events with altered coagulation markers

pathophysiological outcomes. Nevertheless, whether the generated alterations in the organs distant from the exposure site are due to the direct impact of *translocated NPs or particle-induced inflammatory and oxidative stress* at the exposure site is still being investigated. Extensive exposure to NPs occurs through the respiratory system, oral route, and skin. Increasing the use of Ag NPs in

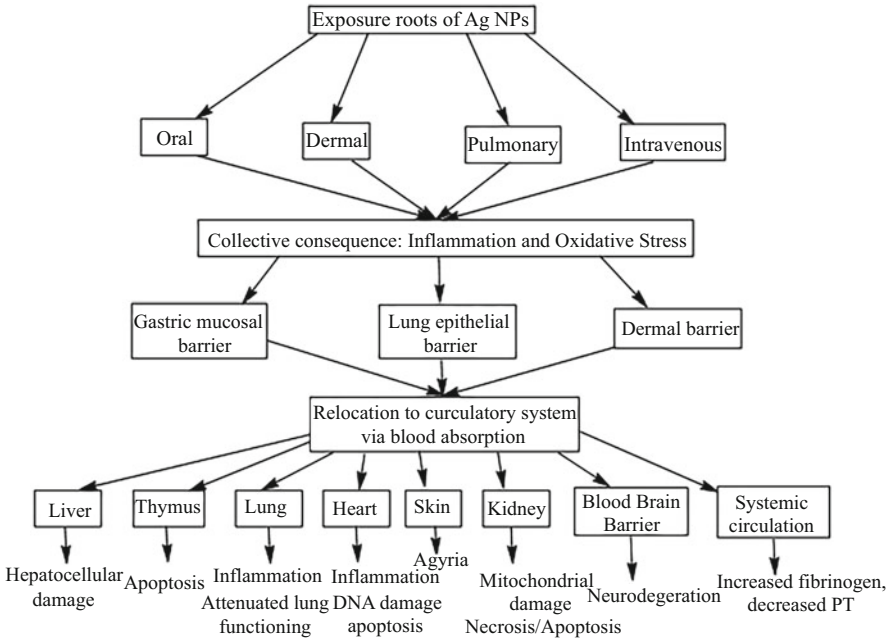

Fig. 19 Schematic illustration of Ag NPs biodistribution and toxicity following various exposure routes

healthcare products like soaps, creams, antiseptic lotions, and sunscreens significantly enhances the risk of occupational exposure of these NPs to the working employs.

- Regarding respiratory exposure, a recent study screened the effluent from a commercial Ag nanowashing machine and showed that Ag NPs at an average limit of 11 $\mu g \cdot L^{-1}$ were released into the environment. Similarly, an occupational study in a silver manufacturing plant revealed a significant increment of Ag NPs in the air during production, resulting in a peak area concentration of more than 290 $\mu g \cdot m^{-3}$. Recently, a subchronic rat inhalation toxicity investigation revealed 0.19 $\mu g \cdot m^{-3}$ as the occupational exposure limit for Ag NPs, via consideration of human equivalent concentration. Following inhalation, the transport and deposition of Ag NPs are nonuniform and are affected by several factors such as flow rate, airway structure, pulmonary function, age, gender, and most importantly, the characteristic particle size. In general, particles less than 0.1 μm in diameter have a deeper penetrating ability into the alveolar region via diffusion. Owing to this, the clearance of NPs takes longer which complicates the NPs-tissue interactions with manifold altered pathophysiological impacts. A <0.1 μm diameter results in easier translocation of the NPs in the alveolar-capillary barrier which influences lung homeostasis.
- Oral exposure to Ag NPs is facilitated by their presence in dietary supplements, water contamination, and fish consumption. Recent studies have inferred an oral

exposure risk of Ag NPs from their migration across the food packaging to the food which is to be consumed. The inhalation route aggravates the oral exposure as the NPs cleared across the mucociliary escalator, being swallowed and cleared across the gastrointestinal tract (GIT). Estimates predict a daily consumption of Ag NPs in humans via ingestion as around 20 to 80 μg. After ingestion, the *GIT serves as a mucosal barrier to promote a selective degradation and uptake of nutrients (including carbohydrates, peptides, and fats)*. The NPs can act on the mucus layer, followed by translocation *to the bloodstream and ultimately accessing each organ after the epithelial invasion*. The uptake of <100 nm NPs happens via endocytosis which being within enterocytes, aggravates oxidative stress, DNA damage, and inflammation.

- Human exposure to Ag NPs can also happen via the dermal route, reportedly the first line of defense between external and internal environments. The ability of solid NPs to penetrate healthily and breached human skin as well as their diffusing ability to the underlying structures is well-studied. Chance toxicity of Ag NPs (as drug carriers) aggravates from their intravenous and intraperitoneal administrations that release them directly into the systemic circulation. Clearance of NPs delivered through these distinct routes forms a major basis of toxicity wherein studies demonstrated a clearance from human organs after a recovery period is typically between 17 days and 4 months. Toxicity further enhances *in tissues like the brain and testes*, which have been noted for the persistence of Ag in the long-term exposure studies, inferring a difficulty in systemic clearance.
- The physiological actions of Ag NPs have been examined discretely through in vitro and in vivo settings, wherein the former has revealed toxicity on multiple cell lines, including those of macrophages (RAW 264.7), bronchial epithelial cells (BEAS-2B), alveolar epithelial cells (A549), hepatocytes (c3A, HepG2), colon cells (Caco2), skin keratinocytes (HaCaT), human epidermal keratinocytes (HEKs), erythrocytes, neuroblastoma cells, embryonic kidney cells (HEK293T), porcine kidney cells (Pk15) and monocytic cells (THP-1). One-to-one discussion of all these studies is beyond the scope of this chapter theme, readers are thereby advised to refer to the more specific literature. A common aspect of all these investigations pertains to the toxicity of NPs via elevated oxidative stress and inflammation. In one of these attempts, an inspection of A549 NSCLC cells exposed to increasing concentrations of Ag NPs till 24 h revealed significant morphological changes characterized by cell shrinkage, certain cellular extensions, confined scattering manners, and dosage-varied cell death. Another worthwhile attempt revealed DNA damage with overexpressed metallothioneins following treatment of A549 LC cells with 20 nm Ag NPs (0.6 nM concentration on a time-lapse of 48 h). Exposure of rat alveolar macrophages to 15, 30, and 55 nm sized Ag NPs deciphered NPs size-based changes, after being incubated with hydrocarbon coated Ag NPs.
- Regarding the in vivo studies, the Ag NPs are reported to enter blood upon gaining entry via inhalation, ingestion, and intravenous or intraperitoneal injection. Tables 3 and 4 describe the major studies for Ag NPs toxic responses corresponding to their inhalation and intraperitoneal injections. It is clear from

the described studies that the toxicity of Ag NPs is regulated by their characteristic size and capping layer. A lower size easily penetrates the blood vessels and capillaries and is furthered via enhanced circulation within the bloodstream. An interesting observation pertains to the *enhanced inflammatory and oxidative stress-driven physiological compulsions* despite not being traced as deposited in any of the organs. The exclusion from the body is mainly regulated via macrophages (housed in the liver), kidneys, spleen, and feces, which are frequently reported as deposition organs. For the organs like the brain, intestines, and stomach, the barriers of the *bloodstream hinder a NP's escape*. It is often cumbersome to have a reliable prediction of coated and engineered NPs, where the surface coatings can be involved in complex interactions that *prolong the intracellular stay of NPs and delay their elimination*. As a result, there is a greater likelihood of aggravated oxidative stress and inflammation excess (such as necrosis) responses. So, the risk assessment and safer administration of Au and Ag NPs in their drug delivery applications is a challenging task that mandates their thorough structural inspection. Modeling of interdisciplinary parameters such as particle sizes, administration route, nature of the surface coating, specific shape, and the dosage extents form interplay herein, with computational predictions as reliable predictors.

7 Future Prospects and Summary

Efforts to obtain improved response through Au and Ag NPs delivered drugs continue to be a thrust area of research among the pursued medical sciences agendas. The driving forces for NPs include their shape and size modulated chemical and physical activities along with their functionalization attributes. Interest in pursuing a robust functionalization has swiftly relocated the focus toward graphene and its derivatives. With a large SA and versatile geometry, the graphene structure is a worthwhile asset to tap for exploring the binding of biomolecules and receptors. Irrespective of covalent or ionic forces being mitigated for controlling this binding, there is always an important role of SA and stacking forces. The self-assembly of graphene and its derivatives allows motions and optimum conversion of cohesive forces to intermolecular forces. A larger SA results in a higher contribution of basal platform whereby the least energy is required from outside to deliver sustained activities or needed chemical combinations. The efficient scattering abilities and ability to be functionalized with biocompatible carbohydrates such as chitosan, glutaraldehyde, polycaprolactone, and several others, makes graphene a highly robust and useful functional platform for drug delivery. About cancer cure, delivering drugs accurately and timidly always assumes a place after a thorough and exact diagnosis. So, timely diagnosis of tumor development along with implicit stage identification is a highly essential task for improving the success of drug delivery to tumor cells. Perhaps, this is amicably reflected through the terminology distinction and use of the phrase, "theranostic" for simultaneous therapy and diagnostic measures. Advances in combinatorial sciences with the availability of larger functional

SA have provided several new platforms and molecules that could be engineered for need-based response in varying biochemical environments. The emergence of nanofibers and thorough characterization of diverse shapes in NPs (such as *nanodiamonds, nanospheroids, nanocages*, etc.) have been the outcomes of such assets. Gradually, self-assembly-driven molecular interactions are being engineered to obtain an integrated response and add up the individual abilities of each constituent. This is realized in a drug delivery cargo, where the targeting mediator is always accompanied by a separate drug delivery agent. To enhance the societal acceptance and practical usefulness of NPs-mediated drug delivery, the reproducibility attempts must be enhanced. Regulatory trials must be robustly implemented and a thorough matching of delivered drug dosages and NP configurations concerning cancer severity and stage must be maintained. Ethical approvals for animal and clinical trials must be given priority so that a transition to the next subsequent phase can be made without undue delay. This is because the *biochemical and biophysical developmental states* of a tumor cell change with varying stages and each stage is distinguished by distinct receptor expression at the surface. Thereby, the drug concentration and type effective at stage I corresponding hallmarks might not be efficient for further stages. There are many successful in vitro attempts that have >90% feasibility of successful in vivo replication but the *delays in completing formalities and inadequate infrastructural facilities* hinder their laboratory to social transition. Dosage monitoring is swiftly emerging as a key factor in ensuring the stage-specific drug delivery response in the tumor cells.

Mortality from lung and breast cancers is affecting every nation across the globe. The scenario presents an urgent concern for under-developed nations that can't afford expensive *treatments and diagnostic techniques*. So, considering cancer as a threat to mankind as a whole, it seems quite logical that unified cooperation and a determined spirit for preserving mutual harmony can curb this menace. Healthcare funding in such countries must be enhanced and should be a part of *periodic progressive discussions* each time an international delegation meets. Although it is much easier said than done, a constant fearfulness of cancer as a curse to humanity with an unresolved mindset is the only determined way out on a mass scale. Cautionary initiations could be made by recruiting volunteers from these countries as it is better to die painfully than have a manageable living. Access to facilities is not the sole criterion as for LC, the United States remains in the top three of worst affected global economies for the past several years. It is therefore highly urgent to *review and monitor our lifestyle* too and patterns of illnesses must not be taken casually. Dependence on synthetic and processed foods must be reduced along with strict guidelines on adulterating the natural outputs. Respecting and believing the nature is a vital requirement for instilling peace and harmony along with a feeling of living happily with each other. One always ponders as to God who created us never thought of deceiving anyone, giving same abilities, what then makes us feel so hateful toward one another. It is no longer a surprise that today; chronic illnesses are largely caused due to stress. Therefore, we have to search for a preventive than a curative measure as that is hidden in our everyday routine, work management, and how we perceive our surroundings.

8 Conclusions

Delivering drugs using Au and Ag NPs indeed presents manifold aspects to contain the mounting lung and breast cancer mortality. The shape and size-dependent physicochemical activities of NPs along with their enhanced functional SA-driven drug adsorption and targeting-driven engineering are the distinguishing aspects from the conventional delivery approaches. The preparation methods using multiple resources including the parameter varied change in morphology contribute to performance guiding controls in NPs although drug delivery necessitates maintenance in the monodispersed state. Understanding of Au-NPs toxicity is not as clear as that of Ag which deserves timid attention. The robustness of functionalization and self-assembly-driven molecular interactions are the backbones of a successful drug delivery approach. To make the findings significant for future attempts, predictive outcomes must be considered which mandate the involvement of computational biochemists. Benchmark standardization should be practiced with utmost attention to strengthening each new trial based on the previous findings and reproducibility of results via matching the databases. Understanding the physical meaning of drug delivery and the corresponding strategies for maximizing drug internalization sheerly within the tumor cells must be stressed for every new trial.

9 Cross-References

► Nanomaterials: Compatibility Towards Biological Interactions

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Mammalian Cells: Reliability as Model System in the Ecotoxicological Evaluation of Environmental Stressors

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_22-2

Abstract

Disturbance in ecosystem balance is increasing drastically due to human interferences. Different kinds of toxins are continuously being released into the environment from various sources; few are naturally produced in environment like cyanotoxins, and the rest are released from industries or other human activities. Living organisms, including humans, come in contact with various kinds of toxins during their lifetime. To understand the level of toxicity and mechanism of action, ecotoxicological evaluation of these environmental stressors is necessary. Selecting/creating an ideal model system for toxicity screening is a major concern among scientist. At present, various animal models like mice, rats, fish, etc., and different cell lines are used to understand the toxicological potential of environmental stressors. Mammalian cell lines are widely used to predict the toxic effects on cellular level, especially on human perspective. To understand the mechanism of toxicity at organism level, other models like fish, mice, rats, etc., are used. This chapter reviews the benefits and drawbacks of different model system like teleost, algae, and mammalian animal models over mammalian cell lines. This chapter will also help to understand the vitality of these model systems in toxicity evaluation and choosing the suitable ones for ecotoxicological evaluation of environmental stressors.

Keywords

Ecotoxicology · Environmental toxicants · Animal models · Mammalian cell lines · Teleost · Algae · Model organisms

1 Introduction

Living organisms, including human beings and other organisms, during their lifetime, come in contact with diverse environmental and chemical toxicants, such as, xenobiotics, persistent organic pollutants, drugs and pharmaceuticals, etc. These toxic environmental pollutants not only cause potential cellular, tissue, and organ damage, respiratory and cardiovascular disorders, but also pose serious threat to the viability of the organisms (Kelishadi et al. 2009). These pollutants bear mutagenic, carcinogenic, or even teratogenic effects on the germ cells, embryonic and adult stages of all living organisms, and not only induce embryotoxicity but also developmental deformities of the offsprings and the adults, perinatal disorders, and mortality, thereby indicating trans-generational effects, persisting from one generation to the next.

1.1 Major Environmental Stressors and Their Toxicological Effects on Biological Systems

Nowadays, the deterioration in the physicochemical and biological quality of the environment due to the exposure of different types of environmental stressors has become a worldwide problem. Their harmful impacts are observed in both terrestrial and aquatic environmental systems and also on the biota residing in those systems. These environmental stressors are largely capable of inducing potential damage from cellular to organ level of the organisms, giving rise to different types of physiological disorders. These environmental stressors which have devastating effects on the biota are itself the cause of majority of anthropogenic sources like heavy metals (Hg, Pb, Cr), plastics (micro/nanosized), pesticides, fertilizers, PPCP (pharmaceuticals, antibiotics, personal care products), etc., as well as some natural ones like cyanotoxins (MC-LR, cylindrospermopsin, anatoxin), etc. Alarming rise in the rate of urbanization, industrialization, and anthropogenic activities are some of the major causes of these environmental stressors in the environment, which have been piled up for decades. After being released in the environment, they persist in the environment for a long period of time and in comparatively higher concentrations than their natural levels. Elevated concentrations of these environmental stressors in the environment have a wide range of negative impacts on the organisms dwelling in the particular system like animals, plants, fish, lower organisms, microbial species, as common biota (Wasi et al. 2013). Constant exposure to these environmental toxicants for a long period of time induces cellular and morphological changes, DNA damage and breakdown, and genetic disorders, culminating in different genetic disorders like autism, Parkinson's disorder, etc., in human beings. They also induce several organ level toxicities like cardiac and digestive disorders, hepatotoxicity, neurotoxicity, ocular toxicity, and numerous developmental disorders. They also pose the threat of inducing mutational changes, tumorigenesis, and cancer, which have even proved to be fatal to life (Fig. 1).

Therefore, a model system/representative for the identification and evaluation of the toxicological potential of these environmental stressors is of great concern. The ideal approach of ecotoxicological evaluation of environmental toxicants on human beings and on the environment would itself require an extremely large number of human subjects, the actual representatives, who are exposed to these toxicants unknowingly or knowingly under realistic conditions. Assessment of the possible toxicological effects would only be fruitful using these impossible and unethical human subjects. Deviations from these unrealistic and unethical experimental conditions will only approximate the real and ideal situation of the study, i.e., represent a "model system."

At present, a wide range of *in vivo* and *in vitro* model systems are used for the ecotoxicological evaluation of environmental toxicants (OECD 2008), among which the mammalian models include whole animals like mice, rats, guinea pigs, etc., and the *in vitro* cell lines, which have achieved potential attention in toxicological research due to their closeness to human beings. Also, different model systems like teleosts and algal model systems have received much attention in the field of

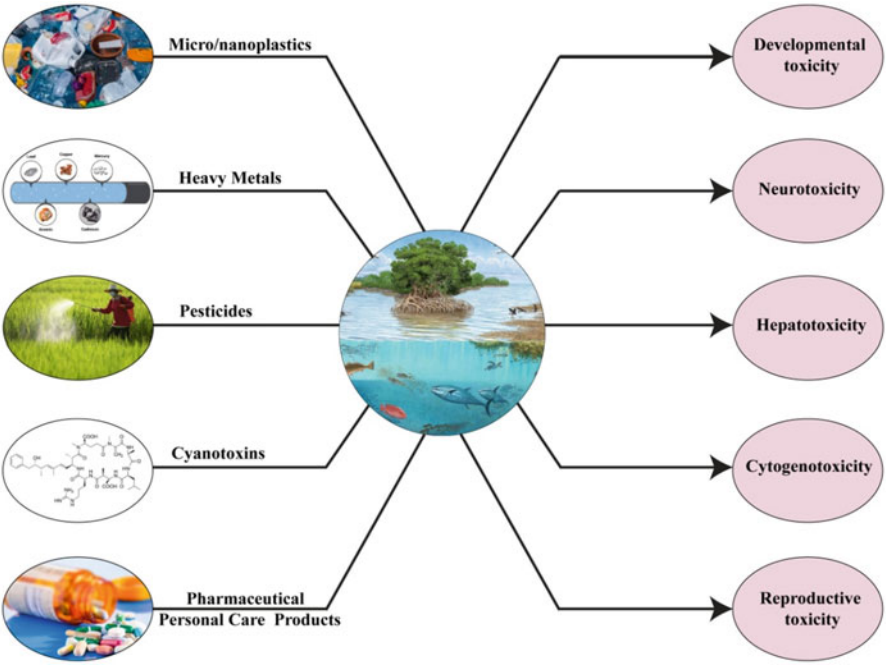

Fig. 1 Major environmental stressors and their toxicological effects

toxicological studies. However, these model systems also face certain challenges and drawbacks in terms of toxicological evaluation of potential toxicants, as they cannot completely mimic human beings in respect of some species-specific toxicological effects of the toxicants being used. Again, simultaneous increases in different animal models and cell line systems day by day are facing inadequacy in standardization of experimental procedures, which in turn impacts the fruitfulness of the model in toxicological evaluation of diverse environmental toxicants (Fig. 2).

1.2 Vitality of Laboratory Animal Models in Toxicological Evaluation of Environmental Toxicants

Nowadays, millions of animals are used in toxicological potential analysis of environmental toxicants. A large increase in animal model use has intended to fill the knowledge gaps regarding the toxic potential of diverse toxicants used worldwide and gained similar effects when compared with human subjects (Table 1).

A vital question now arises on the vitality of the use of mammalian laboratory animal models, as a large sum of money is being spent on the maintenance and experimentation of toxicological analysis of toxicants. Besides these, the laboratory animal model encompasses certain limitations and drawbacks, few of which are discussed as below.

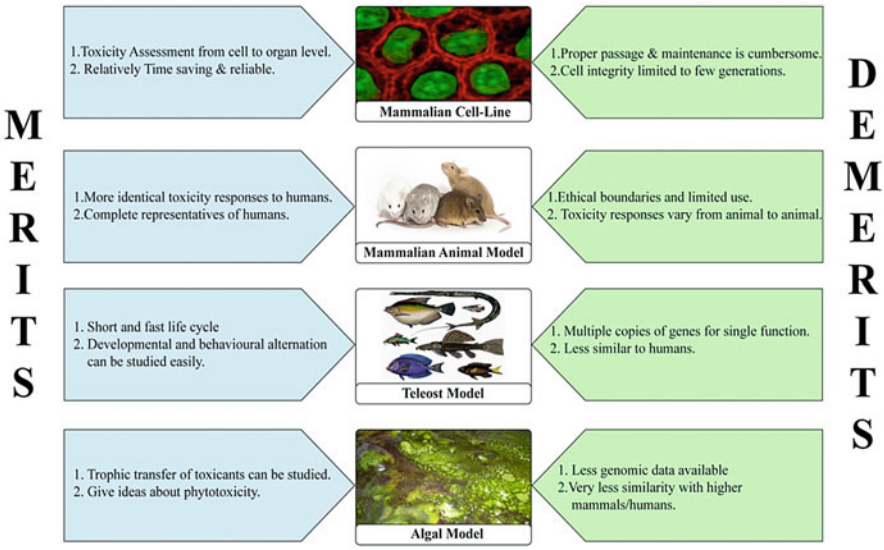

Fig. 2 Merits and demerits of model systems in ecotoxicological evaluation of environmental stressors

1.3 Limited Toxicity Responses Identical to Human Subjects

The first demerit of mammalian animal models reflects its limitations to mimic identical human responsiveness to a toxicant. The human body is comparatively different from laboratory mammalian animal models and has different physiological and metabolic repertoire, immune and adaptability responses to disease conditions. A study conducted shows that some toxic chemicals that are potential irritants to rabbit skin pose no irritability effects on human subjects, as depicted by "skin patch test" (Basketter et al. 2004). However, evaluation of toxicological potential in different animal models, using the same toxicant, gives more or less identical results, which signify that different animal species show similar responses to each other to some chemicals (approx. 50–60%), compared to a lesser extent they depict those of human beings (Gottmann et al. 2001; Schardein et al. 1985).

Therefore, utmost care must be taken while doing toxicity assessment of environmental toxicants using animal models. Toxicological evaluation must often be carried out simultaneously using two animal models; if no toxic effects are observed in one model organism, then the second model organism must be tested to improve the vitality of the toxicological evaluation of the environmental toxicants. This not only increases the efficacy of the evaluation assay but also increases the potential to determine the sensitivity of the toxic agent to humans.

Table 1 Toxicity studies of environmental toxicants using laboratory animal models

Model	Environmental toxicant	Dose used	Exposure period	Toxicological effects	References
Mice	Bisphenol A	50 µg, 5 mg, and 50 mg	7–14 days of gestation	Impaired spermatogenesis, impaired fertility (multigenerational effect)	Rahman et al. (2021)
Sprague–Dawley rats	Cadmium (Cd)	0.6 mg	12 weeks	Accumulation and dysfunction of pancreatic islets, hyperglycemia	Fitzgerald et al. (2020)
Mice	Cyproconazole and prochloraz	50–500 ppm	28 days	Hepatotoxicity	Marx-Stoelting et al. (2017)
Wistar rats (outbred)	MC–LR and cylindrospermopsin	7.5 + 75, 23.7 + 237, 75 + 750 µg	0, 24, 45 h	Genotoxic and histopathological damage in liver	Díez-Quijada et al. (2020)
Rabbit (male)	Lead	0–3.85 mg	15 weeks	Reproductive toxicity – (spermiation inhibition)	Moorman et al. (1998)
Wistar rats	Polystyrene microplastics	0.5 µm	90 days	Pyroptosis and apoptosis of ovarian granulosa cells	Hou et al. (2021)
Mice (C57BL/6 J)	Dioxin	5 µg	6 weeks	Liver fibrosis development	Duval et al. (2017)
Wistar rats	Iron oxide nanoparticles	10 mg	4 days	Neurotoxicity	Fahmy et al. (2020)
Mice	Polystyrene microplastics	500 µg/L	28 days	Acute colitis, inflammation, and lipid disorder in liver	Zheng et al. (2021)
Rabbit	Cd, Pb, Hg (heavy metals)	2.3, 4.1, 30 mg (respectively)	28 days	Hyperchromic macrocytic anemia, liver damage and pancreas toxicity, tubular nephrosis, etc.	Bersenyi et al. (2003)

1.4 Assessment of Multiple Toxicity End Points on Small Population Size

The second important issue regarding the use of laboratory mammalian animal models is the toxicological testing of multiple end points, over a small population size, which inevitably contributes to a huge number of false positive results, as positive associations are always observed in multiple toxicity analyses.

There are numerous long-term toxicological studies, where animal models are exposed to certain toxicant or environmental stressor for a longer period and observed for multiple organ effects after the exposure period (Barai et al. 2017; Aktar et al. 2017). In such cases, it is observed that some toxicological end points are positive (false positive), owing to the small group sizes (n). This is an indicator of statistically insignificant results, as analyses of the multiple variables in small populations cause extreme clustering of results in the multiple same results. At the same time, it should be kept in mind that a large sum of money is invested for toxicological analyses of environmental stressors, so, increase in population size on a large scale, to avoid false positive results, is unrealistic as well as undesirable, from ethical and welfare perspective of the animal subject. Therefore, all the positive results, including the false positive ones, are recorded as true positive results, which make the evaluation less desirable and less authentic for risk assessment of environmental toxicants.

1.5 Improper Study Design and Dose Determination

Another important issue faced with toxicological testing of environmental toxicants using animal models is related to the proper experimental study designing, where a high precautionary (conservative) approach is presently employed.

To limit the large number of animals used and the cost associated with maintenance and toxicological analyses, the study is usually carried out with the previously determined, maximum tolerable dose of an environmental toxicant. Such predetermined doses of toxicant seem to be 1000 fold or higher, as compared to the actual intended dose for human beings (in terms of mg/kg. of body wt.), owing to the size differences, as human beings are not 70 kg rats/mice.

This strategy of improper toxicant dosing and faulty experimental designing and its impact analysis on animal models not only diminishes the reliability of the study but also the correlation of toxicity assessment and evaluation between animal models and human beings (Rietjens and Alink 2006).

1.6 Prevalence of Toxicological Effects on Human Health

One of the major concerns is the prevalence and toxicity analyses of the potential toxicant on human health, i.e., how many environmental toxicants really do have hazardous and detrimental effects. It is observed that approximately 80% of the

potential environmental toxicants which pose serious toxicity to the health of human beings and are regarded as well as registered as potential hazardous toxicants are currently showing no acute toxicity in current toxicological analyses, after a long time span.

Many of the proposed and well-established biological and physiological effects of the toxicants have vanished or they rather depict mere toxicity, as shown by the previous toxicological studies. So, it is clear that the toxicological potential of rare potential environmental toxicants, done with improper model organisms with imperfect standardization of the technique, shows mere similarity with those of human beings. This raises the probability of yielding false positive results (Bremer et al. 2007). Any laboratory which later requires them to reproduce the same presumed toxicity to validate an alternative approach cannot do so, as false positive results are difficult to be reproduced again, when a different test is being conducted using the same environmental toxicants.

These approaches unnecessarily restrict the use of many substances and therefore create devastating fear among other common people about previous exposure and indirectly lead to expensive ways to virtually replace these environmental toxicants with proper alternative ones (Fig. 3).

2 The "3R's Principle": An Alternative Approach to Laboratory Animal Use

The *3Rs – replacement, reduction, and refinement*, which signifies *replacement* of animal models with nonanimal ones, *reduction* of the vast number of animal subjects used, and *refinement* in order to reduce the pain and suffering of the laboratory animals used, as first proposed by Russell and Burch in 1959 – are the most universally accepted fundamental ethics in good lab animal practices (Fig. 4).

Russell and Burch (1959) stated that "*Refinement is never enough*, *and we should always seek further reduction and if possible replacement …. Replacement is always a satisfactory answer.*"

A broad range of other nonanimal mammalian model systems are potentially replacing the conventional laboratory animal use for toxicity assessment of environmental stressors, where *in vitro* mammalian cell cultures, i.e., the "cell line" system is the most authenticate as well as the reliable one.

3 "*In-Vitro* Mammalian Cell Line": The Most Reliable Alternative of Animal Model in Toxicological Assessment of Environmental Toxicants

Ecotoxicological assessment of potential environmental toxicants on humans requires an average bulk of laboratory mammalian animal models, which is getting out of the scope for waivers day by day. As a result, there is a growing interest in

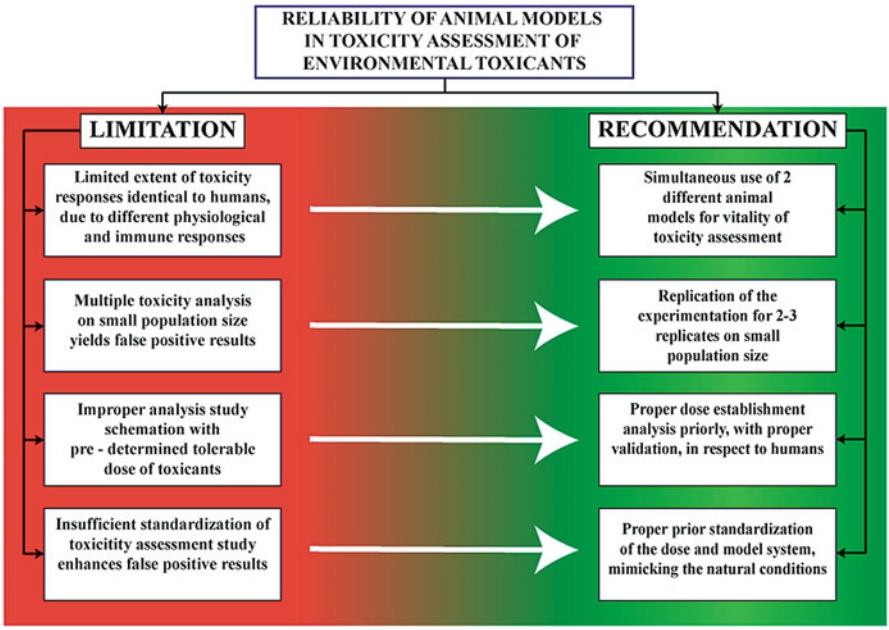

Fig. 3 Major limitations and ways to alleviate the limitations associated with animal model use in ecotoxicological evaluation of environmental stressors

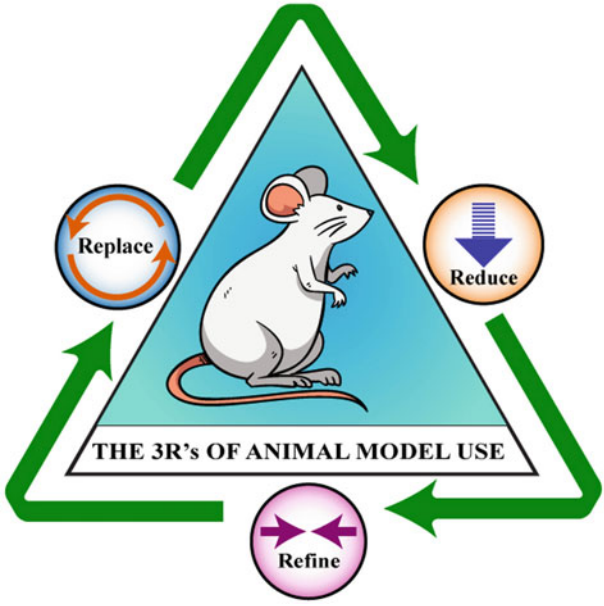

Fig. 4 The 3R's principle of animal model use in research by Russell and Burch (1959)

nonanimal-based mammalian alternatives to humans for toxicity assessment of environmental stressors (Table 2).

Thus, the most reliable and authentic alternative to these laboratory animal models are the "*in vitro* mammalian cell lines," which are not only feasible but also have less boundaries as compared to the former.

Different types of *in vitro* mammalian cell culture systems, including primary cell lines, immortal cell lines, secondary cells, stem cells, tissue culture, organotypic culture, etc., are used on a wide range basis for ecotoxicological evaluation of environmental stressors, on multiple end point evaluation analysis. These *in vitro* mammalian cell line models are feasible and have less limitations to be used on a large-scale basis as an alternative for toxicological analyses of environmental toxicants (Rim 2019). Some advantages of using an *in vitro* mammalian cell culture model for ecotoxicological evaluation of environmental stressors are discussed ahead.

3.1 Instant Assessment of Basal Cell Structure, Function, and Viability as Toxicity Index

In vitro mammalian cell lines have made it possible to estimate the toxicological potential of environmental stressors at the preliminary level by assessing the basal cell structure and function and death (Vinken and Blaauboer 2017).

The initial and most readily observable effect is morphological change and the cell-shape alteration and loosening in the cell monolayer as induced by the toxicant. Modification or changes in cell shape and morphology are easily denoted by cell vacuolization/blebbing out, which can be easily observed by microscopy and SEM/TEM to observe its fine ultrastructural details.

Decreased cell growth, as indicated by the slower rate of cell-monolayer formation, is another easily observable index of toxicant-induced toxicity. Evaluation of the ID50 or LD50 of the toxicant on the cell and other growth parameters is easily assessed.

Toxicant-induced cell death or apoptosis, as determined using the "dye-exclusion technique," is another major index of toxicity. Microscopic counting of the dead/live cells in the presence of the toxicant and its comparison with no toxicant-induced "control" provides the "lethality index" of the environmental toxicant; it can also be assessed by MTT assay, XTT assay, etc. (Schneider et al. 2017).

Altered plating efficiency, i.e., the monolayer formation rate, induced by the toxicant within a particular time span, efficiently indicates the toxicity on the cell survival and reproductive ability. It can easily be detected by some simple assays like cell counting, DNA/RNA and protein estimation, radiolabeling or fluorescent tracing of biochemical components.

The toxicological potential of the environmental stressors, as indicated by altered basal cell activity, can also be easily assessed instantly by observing changes in metabolic or biochemical pathways by gene expression studies or radio-tracing, changes in ATP levels by luciferase assay, cell damage by acid phosphatase assay,

Table 2 Toxicity studies of environmental toxicants using *in vitro* mammalian cell lines

Cell line (*in vitro*)	Environmental toxicant	Dose used	Exposure period	Toxicological effects	References
HCT – 116 (human colon carcinoma)	Bifenthrin	5–200 μM	24 h	Disrupted mitochondrial function, increased ROS and oxidative stress	Bouaziz et al. (2020)
PBMC, HeLa, KATOIII, HDF	Polystyrene microfragments	10, 100, and 1000 μg/mL	1–4 days	Physical cell damage, cytotoxicity, and ROS generation	Choi et al. (2020)
CD34+ (hematopoietic progenitor cells)	Chromium and cadmium	0.1 μM and 10 μM (respectively)	48 h	Autophagy	Di Gioacchino et al. (2008)
FL cell line (human amnion epithelia)	Bifenthrin	0, 5, 10, 20, and 25 mg/L	4 h	Enantioselective cytotoxicity	Liu et al. (2008)
NRK – 52E (renal cell line)	TiO_2 nanoparticles	20 μg/mL	24, 48, 72, and 96 h	Renal cell cytotoxicity and apoptosis	Valentini et al. (2017)
SH–SY5Y (neuroblastoma)	MC–LR, cylindrospermopsin and mixture	0–100 and 0–10 μg/mL	24 and 48 h	Neurotoxicity	Hinojosa et al. (2019)
iPSC (human-induced pluripotent stem cell)	Chlorpyrifos	30 μM	8 days	Neurodevelopmental toxicity; inhibition of neural induction	Yamada et al. (2017)

cell membrane damage, as indicative of cell death by lactate dehydrogenase assay, which can easily be done by using the cell culture medium used for cell growth.

3.2 Toxicity Assessment on Specialized Cells as Indicative of Tissue or Organ Damage

Organotypic culture of the tissue culture, mimicking some specialized functions, can also be widely used *in vitro* to evaluate the toxicity of eco-toxicants at the tissue and organ level (Mossoba et al. 2020). These types of *in vitro* cell systems also have some specialized functions (like glycogen metabolism in hepatocyte cell lines, heart beat rate in cardiomyocytes, and phagocytic potential of monocyte/macrophage cell lines) specific to an organ in spite of the basal cell function. Therefore, structural alteration and changes in specialized functions are clear preliminary indicators of tissue or organ level damage (Kwon et al. 2019).

3.3 Ease of Prediction of the Toxicant's Mechanism of Action

Most of the toxicity of the environmental toxicants relies upon their interaction with cellular macro- and micromolecular components of the organisms. This in turn alters the activity of the biological molecules and thereby impacts changes in the biochemical and cellular pathways as a response to the toxicity (Reventun et al. 2020). The ease with which cellular macro- and micromolecules can be isolated and characterized makes it possible to predict the broad spectrum of toxicity end-points and its mechanism of action through evaluation of gene and protein expression studies, downregulation or upregulation of cellular signaling pathways.

3.4 Predetermination of the Toxic Potential at Concentration and Exposure Conditions of the Toxicants

Evaluation of toxicity by using *in vitro* cell lines helps in determining the concentration of toxicity and exposure conditions at which the toxicant gains its ability to induce lethal toxic effects on human beings. The toxicant can easily be added to the culture medium of the cell to assess the toxicity, which assures the proper cell exposure (Aziz et al. 2014). The concentration and the exposure conditions at which the toxicant induces its maximal toxicity can be predetermined to appropriately mimic the scenario in human exposure.

The toxicant can be easily exposed to the particular *in vitro* cell by adding it to the culture medium depending upon the physicochemical properties of the toxicant (Sambale et al. 2015). Toxicants that are hydrophilic in nature can be directly added as they get completely dissolved in the aqueous part of the culture medium; hydrophobic and lipophilic toxicants can be alternatively added by DMSO. Special

equipment is also present for constant exposure of gaseous pollutants or fumes for evaluation of their ecotoxicological potential.

In order to obtain enhanced, comparable, and valid toxicity results with those of the human subjects, the toxicological evaluation procedures must priorly be standardized by employing different experimental approaches and procedures. Again, well-defined and characterized cell lines of human origin must be taken, as it is important to determine the toxicity up to a higher level of organisms. So, the best and most appropriate cell line system and suitable assay method should be relied upon to assess the ecotoxicological potential of environmental toxicants.

3.5 Preliminary Characterization of the Metabolism and Biotransformation of the Toxicants

The most hazardous and detrimental part of the ecological toxicant exposure is that not only the negative impacts on health have to be assessed, but it is also necessary to assess how the toxicant is metabolized or biotransformed to other metabolites and how long does it persists inside the body. The *in vitro* cell line has made it easier to assess the metabolism of the toxicant and its biotransformation to other metabolites, which also acts as potential toxicants and in turn may also pose threats to the cell, even more toxic than their parent toxicant. Some toxicants are easily metabolized, whereas some persist in cells for a longer time and cannot be easily metabolized or even flushed out of the cell, showing long-term toxic effects to the cell, which are also easily determined by the *in vitro* cell line. Therefore, understanding of the metabolism, distribution, biotransformation, and ultimately excretion of the toxicant is of utmost importance, where the mammalian cell line is increasingly gaining importance, whereas *in vivo* animal models are only used for toxicity assessment of the environmental toxicants (Andersson et al. 2012).

3.6 Relatively Time-Saving and Effective Way to Assess the Priority Ranking of the Toxicant

In this decade, a large number of environmental toxicants are being produced and introduced into the environment by various anthropogenic, environmental, and physicochemical processes. Exposure to these environmental toxicants in even microgram quantities can cause serious health problems to terrestrial and aquatic animals. The toxicity studies of most of these toxicants are inadequate and even nonexistent for some other toxicants. The toxicological assessment of these toxicants became easier as well as time-saving by employing "*in vitro* cytotoxicity" using mammalian cell lines (Sharma et al. 2019). At the same time, potential ranking of these toxicants as compared to other chemicals became possible. In vitro cytotoxicity assessment also suggests some additional toxicity analyses to be performed, which serve as a useful basis for priority ranking of the toxicant in a similar class of environmental toxicants according to their potential toxicities.

4 Negative Aspects of *In Vitro* Mammalian Cell Lines in Ecotoxicological Evaluation of Environmental Stressors

The *in vitro* mammalian cell lines, apart from their advantages in ecotoxicological evaluation of environmental toxicants, also face some major limitations. The major one being susceptibility variation to a particular toxicant in a cell population. Also, proper maintenance of cell culture relies upon cryopreservation techniques and challenges faced in prediction of comparison in the *in vivo* to *in vitro* dose-response and risk relationships (Hawksworth 1994).

The mammalian cell line can assess the toxicity at the basal cell function, which also supports the specialized cell function, so toxic alteration of the former somehow affect and alter the specialized function. Therefore, proper toxicological assessment of tissue to organ level damage cannot be assured in all toxicity analyses.

The effect of environmental toxicant on the *in vitro* mammalian cell may somewhat differ, depending upon the incubation period and incubation conditions as well as the concentration of the toxicant used. Therefore, unless the experimental design is standardized with respect to particular cell types and validated, it may prove difficult to assess and compare the same toxicological effects to other living organisms and human beings.

Compared to the whole animal model, which is a relatively complete representative of human beings where almost all basal, specific, and organ level toxicities working coordinately can be depicted, *in vitro* cell culture is a mere representative model of a particular cell or tissue in the body and mimics only the response of the cell to the toxicant, not the complete organism, working in a coordinated way.

The cell culture system merely represents a complete organism, as well as being difficult to maintain. They hardly represent the complete scenario of the toxicity assessment of environmental toxicants up to organ level basis. The cell lines are passaged up to a few generations in secondary cell culture techniques, so their integrity cannot be maintained up to many generations of passage, which makes them unfit for ecotoxicological evaluation of the chronic toxicity of environmental toxicants.

5 Advantages and Disadvantages of Using Teleost Models over Mammalian Cells in Ecotoxicological Evaluation of Environmental Stressors

The assessment of the toxic effects of an assortment of potential environmental toxicants in animal systems and fish models, besides mammalian animal models and cell lines, is also gaining worldwide attention. Different stages of the life cycle of fish and different exposure routes of the toxicants can be used to assess the toxicity of different environmental stressors in fish models. Fish models are widely used to study early developmental impairments, effects on sex differentiation and reproductive performance, as well as various genetic dysregulation in response to various kinds of environmental toxins or pollutants. Zebrafish (*Danio rerio*), fathead

minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*), and goldfish (Carassius auratus) are few examples of the well-known fish models that are diversely used in various ecotoxicological assessments of environmental stressors (Norrgren 2012) (Table 3).

There are several advantages of using fish models over mammalian cells in ecotoxicological evaluation of potential environmental toxicants. Fish models are very easy to maintain in laboratory conditions and occupy less space due to their smaller size, and they can survive for an extended period of time with little cost of maintenance as compared to mammalian models. Maintenance of fish in the laboratory is cost-effective compared to that of mammalian cell lines. Fish models are easily available, and ecotoxicological studies of environmental stressors using fish models can be easily replicated many times, which confers to the reliability and authenticity to the toxicity assessment of the toxicants. In natural environment, human beings and other organisms contact different kinds and doses of environmental stressors for a long period of time. Due to the longer life span of mammals and the delay in their sexual maturation, it becomes complicated to investigate the multigenerational impacts and long-term impacts of environmental stressors on different stages of their entire life cycle in laboratory conditions. To overcome these problems, fish models are used reliably owing to their shorter life span and quick sexual maturation for toxicity assessment of environmental stressors. Fish like turquoise killifish (*Nothobranchius furzeri*), whose average life span is about 6 months and attains sexual maturity within a 3 month period, can be used as a suitable model for multigenerational toxicity assessments of diverse environmental stressors (Thoré et al. 2021). Zebrafish are also admired as efficient and flourishing models for various laboratory research studies, including ecotoxicological assessment of toxicants upon the health and well-being of different organisms (Sökmen et al. 2020). Zebrafish have several advantages like smaller size, easy maintenance in laboratory conditions, shorter life cycle, and high fecundity rate. Breeding is simple in the laboratory, and each spawning of zebrafish can produce up to 100–200 eggs. The transparency of the zebrafish embryos and larvae allows an extra advantage to easily observe the changes happening in them during toxicant exposure as well as the distribution of fluorescent-tagged toxins like heavy metals or MPs/NPs (Trevisan et al. 2019; Pitt et al. 2018). Zebrafish genomic content is also completely sequenced as well as readily available, and it is observed to have about 70% similarities with the human genome (Howe et al. 2013). All these characteristics make zebrafish an ideal and suitable model organism to study the effects of toxic environmental contaminants on the normal reproductive and developmental processes of humans and other organisms efficiently. Zebrafish models are widely used in the screening and evaluation of behavioral toxicity, reproductive toxicity, neurotoxicity, immunotoxicity, hepatotoxicity, as well as cytogenotoxicity, aided by environmental stressors.

Several behavioral studies have been conducted using fish models to understand the effects of various environmental pollutants on the behavior of fish in different stages of their life cycle. Early stage impairment and behavioral abnormalities in embryo and larval stages as well as in adult zebrafish on exposure to different kinds of toxins have been reported by various scientists (Brun et al. 2019; Velki et al. 2017;

Table 3 Toxicity studies of environmental toxicants using teleost model

Model used	Environmental toxicant	Dose used	Exposure period	Toxicological effects	References
Zebrafish (*Danio rerio*) larvae	Microcystin-LR	0, 0.8, 1.6, and 3.2 mg/L	120 h	Developmental neurotoxicity Decrease in dopamine and acetylcholine (ACh) content Increase in acetylcholinesterase (AChE) activity	Wu et al. (2016)
Zebrafish (*Danio rerio*)	Polystyrene microplastics (PS-MPs)	20 μg/L	7 days	Hepatotoxicity Inflammation and lipid accumulation, and increase in oxidative stress in fish liver	Lu et al. (2016)
Zebrafish (*Danio rerio*)	Copper ($CuSO_4$)	10, 20, and 40 μg/L	30 days	Reproductive toxicity. Decrease in estradiol level in female and testosterone level in males Alteration in expression level of genes related to steroidogenesis in gonad and brain	Cao et al. (2019)
Zebrafish embryo (*Danio rerio*)	Spinetoram	5, 7.5, and 10 mg/L	86 h	Decreased heart rate and growth rate Induced oxidative stress Immunotoxicity Upregulation of IL-6, IL-8, and TNF-γ Downregulation of IL-10	Cheng et al. (2020)
Fathead minnow (*Pimephales promelas*)	Ketoconazole	25 μg/L	21 days	Reproductive toxicity. Decreased egg production and plasma vitellogenin level. Upregulation of CYP11A and CYP17 in gonads	Ankley et al. (2007)

Medaka (*Oryzias latipes*	Silver nanoparticles (Ag-NPs)	1 and 25 μg/L	1, 2, 4, and 10 days	Cytotoxicity and increased oxidative stress Induction of metallothionein (MT) and CYP1A expression	Chae et al. (2009)
Rainbow trout (*Oncorhynchus mykiss*)	Titanium dioxide NPs (TiO_2-NPs)	0.1, 0.5, and 1 mg/L	14 days	Cytotoxicity and morphotoxicity Induced oxidative stress. Increased edema in lamellae and hyperplasia in gill epithelium	Federici et al. (2007)
Goldfish (*Carassius auratus*)	Triazophos	0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, and 2.4 mg/L	96 h	Cytotoxicity and neurotoxicity Increase oxidative stress. Reduction in AChE activity	Liu et al. 2015
Banded gourami (*Trichogaster fasciata*)	Mercury ($HgCl_2$)	50 μg/L	30 and 60 days	Reproductive toxicity. Decrease in gonadosomatic index (GSI) Reduction in estrogen and testosterone level	Guchhait et al. (2018)
Climbing perch (*Anabas testudineus*)	Fullerene (C_{60}) NPs	5 and 10 mg/L	96 h, 4, 7, 15, 30, and 60 days	Reproductive toxicity. Decrease in serum estradiol and testosterone levels Increase in cortisol level	Sumi and Chitra (2020)

Sarasamma et al. 2020). This sort of behavioral study cannot be performed using mammalian cell lines. The ecotoxicological studies using mammalian cell lines will not give sufficient information about the interactive effects on the full body system. Reproductive performance or fitness of organisms depends on various factors like gonadotropins, sex steroid hormones, and stress, and is controlled by the interconnected function of gonads with other internal organs. In order to obtain a complete understanding of the toxicity level of the toxicants on reproductive performance and fecundity, *in vivo* study is more suitable and reliable in comparison to the cell line system. Many environmental stressors can result in genetic mutations or epigenetic changes like DNA methylation, which will affect the exposed organisms as well as their future progenitors. This type of multigenerational toxicity effect analysis as well as effects on future progeny upon parental exposure to any toxins using mammalian cell lines is not possible, which is a major limitation of the cell lines. There are different markers in fish models as well as in mammalian cells to detect the level of toxicity of the potential environmental toxicants. Though the change in the expression of the marker genes in response to toxicant exposure can be studied in both fish models and mammalian cell lines, the outcome of those effects at organ and system level will not be possible to understand completely using *in vitro* methods.

In neurotoxicological studies, zebrafish models are better alternatives to the cell lines as they are more complex as compared to cell lines. Zebrafish have blood-brain barriers like humans, which protect the brain from various toxic effects (Jeong et al. 2008). Using cell lines in neurotoxicological studies, the effects of the blood-brain barrier cannot be understood. Other advantages of using zebrafish are its genetic similarity with mammals and the development of central nervous system and brain within a few days of postfertilization, as a result of which faster experimental results can be obtained for toxicity analysis (Howe et al. 2013; Kalueff et al. 2014).

Both fish models and cell lines provide marker-based and protein-based outcomes in reproductive toxicological studies, but the effects on reproductive success and fecundity cannot be fully understood using cell lines. Using the zebrafish model, the understanding of the effects over the entire hypothalamus-pituitary-gonad-liver axis, reproductive endocrine systems, as well as fecundity and fertilization rates can be assessed with proper validation. Different somatic indices like hepatosomatic index and gonadosomatic index, which are potential markers of environmental stressors, generally measured by comparing the weight of the particular organs (viz. liver, gonads, etc.) with whole body weight, are also not possible using mammalian cell lines. Different epigenetic changes, like DNA methylation, due to environmental stressors in the parent body may also affect the process of offspring development. Mammalian cell lines are not suitable for these kinds of studies, but zebrafish models can be used to understand the effects of toxins or pollutants on offspring on parental exposure (Teng et al. 2020).

Toxins may enter into the body through various routes like absorption via skin or gills, ingestion, or inhalation, and depending on that cause different levels of toxicity. Using mammalian cell lines, the effect of different exposure routes to toxins cannot be evaluated. Using fish models, the study of the effects of environmental

toxins dissolved in water or mixed with the food supplements, mimicking the natural scenario in laboratory conditions, can also be used to study the bioaccumulation of those toxins in different organs. In embryonic stages, accumulation of polystyrene nanoplastics is reported in the eyes, yolk sac, head, and pericardium. During the larval stages, accumulation in the gastrointestinal tract, liver, pancreas, and gallbladder is reported (Pitt et al. 2018; Van Pomeren et al. 2017). In adult zebrafish, it mainly accumulates in intestine, gonads, liver, and brain (Sarasamma et al. 2020). Most of the environmental stressors get transferred to higher trophic level from lower one through food chains, which results in the biomagnifications of potential environmental toxins. Using mammalian cells as a model in ecotoxicological studies, it will be difficult to understand this trophic transfer of toxins through food chains and biomagnifications that are happening in the natural environment. But using fish as a model system, we can mimic these changes of events occurring in the natural food chain artificially in laboratory conditions. By growing algae in the presence of environmental stressors and using them as food source for herbivorous zooplanktons and finally feeding fishes with those zooplanktons, the trophic transfer of these environmental pollutants can be easily assessed (Cedervall et al. 2012).

Besides the advantages of fish models in ecotoxicological evaluation of environmental stressors over mammalian cells, there are several drawbacks too, for which scientists prefer mammalian cells as a model system in various toxicological experiments. To predict the exact effects of any environmental stressors on humans, fully relying on the fish model is unjustified and improper, as they are less similar to human; their physiology is not identical to human. Fish are exothermic animals; their adaptation in nature is different from that of human beings. Toxin exposure routes to embryos and larvae differ from those of humans, because fertilization in fish is external, whereas in humans, it is internal, and the embryo is protected by a protective membrane, which may interfere with toxic effects (Cudd 2005). The prediction of the maternal transfer of toxins to the embryo through the placenta by studying a fish model is also not possible. Fish also have multiple copies of genes for single functions, which react slightly different from each other in response to the same environmental stressors. This may require additional screening to understand the role of each copy of gene completely. The life span of humans is much longer as compared to zebrafish, whose life span is 3–5 years and whose life stages change very fast as compared to that of humans. So the prediction of the effects of toxins in early stages of human development by studying zebrafish larvae is also undesirable. Fertilization in fish is also external with no or very little parental care, and they are much more sensitive to environmental stressors than any other mammalian model or cell lines. The sex differentiation pattern of zebrafish is also dissimilar from that of humans and higher mammals. The gender of zebrafish shows plasticity in the early stages of development and may change depending on the external environment, and therefore, the effects of stressors in human embryos cannot be properly deciphered by studying fish larva. Fish also need water to survive; many water-insoluble stressors cannot be evaluated properly using a fish model in an aquarium (Lardelli 2017). Though fish are great models to evaluate the ecotoxicological effects of environmental stressors, they will not give exact information or ideas about the

toxic effects on humans or higher complex mammals, for which mammalian cell lines are more suitable as well as reliable to evaluate the ecotoxicological effects of environmental stressors on humans and other mammals. Firstly, these cells are from humans or other mammals, which have great similarity with human cells. Mammalian cell lines can be maintained in laboratory conditions for prolonged years. More genomic data is available as mammalian cells have been used in research for years. Tissue-specific effects of different environmental stressors can be observed using mammalian cells from any particular organ. Using cell lines, studying the effects of environmental stressors on cell cycles, chromosomal division, and check points has become extremely easy and practical (Agrawal et al. 2015).

Evaluations of cytotoxicity using mammalian cell lines as a model give more information on the changes associated at the genomic level as well as the protein level. Mammalian cell lines provide more information about the changes in protein folding patterns, protein-protein interactions, and posttranslational modifications in response to environmental stressors (Ebbinghaus et al. 2010). As every animal in this world has some ecological role, using mammalian cell lines as models to evaluate the ecotoxicological potential of environmental toxicants will in turn minimize the use of other live animal models in laboratories, which will be good for the ecosystem and allow the 3R's principle of animal model use in research to be more successful.

6 Advantages and Disadvantages of Using Algal Models over Mammalian Cells in Ecotoxicological Evaluation of Environmental Stressors

The ecotoxicological evaluation of environmental stressors should be implicated on multiple organisms from different trophic level. Algae are the main primary producers of aquatic environment and are very sensitive to the toxic environmental contaminants. Algae are cosmopolitan in nature and are found in marine, freshwater, as well as in terrestrial habitats. Toxicity assessment of various kinds of environmental stressors in the aquatic as well as terrestrial environment can be efficiently performed using algal models (Table 4).

Changes in algal structures and measures are great indicators of water pollutants like microplastics (Wang et al. 2021). As algae belong to the primary trophic level of the food chain, assessment of the trophic transfer of environmental stressors is possible using an algal model. The trophic transfer of polystyrene nanoplastics through the aquatic food chain was observed, beginning with primary producers, single-celled green algae (*Chlamydomonas reinhardtii*), and progressing to primary consumers, *Daphnia magna*, secondary consumers, Chinese rice fish (*Oryzias sinensis*), and finally tertiary consumers, fish *Zacco temminckii* (Chae et al. 2018). Algae grow at a very faster rate and require very minimal maintenance in laboratory as compared to the *in vitro* mammalian cells. Algae have a short and quick life cycle and hence require comparatively less time to evaluate the toxicological effects of environmental stressors (Huang et al. 2016). As algal structures are very simple, ranging from unicellular diatoms to multicellular seaweed, and they can divide by

Table 4 Toxicity studies of environmental toxicants using algal models

Model used	Environmental toxicant	Dose used	Exposure period	Toxicological effects	References
Marine diatoms (*Chaetoceros calcitrans* and *Nitzschia closterium*)	Copper	0–800 μg/L	96 h	Increase in carotenoid content Decrease in chlorophyll a/c ratio Increase in catalase (CAT) activity	Neethu et al. (2021)
Marine diatoms (*Skeletonema costatum*)	Silver nanoparticles	0.5, 5, 50, and 500 μg/L	24 h	Induce ROS generation Decrease cell viability Reduce chlorophyll a content Inhibit photosynthesis	Huang et al. (2016)
Red seaweed (*Sarcodia suiae*)	Cadmium	1 and 5 mg/L	24 h	Decrease in chlorophyll a content Decrease in oxygen consumption and evaluation Increase in phycoerythrin, phycocyanin, and allophycocyanin content	Han et al. (2020)
Marine red algae (*Gracilaria dura*)	Selenium, spermine, and cadmium	0.4 mM $CdCl_2$, 1 mM Spm, and 50 μM Se	4 days	Inhibit algal growth Induce ROS generation Inhibit antioxidant system Enhanced DNA demethylation level	Kumar et al. (2012)
Microalgae (*Scenedesmus obliquus* and *Desmodesmus pleiomorphus*)	Zinc and cadmium	1, 5, 10, 20, 30, and 40 mg/L of Zn 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, and 20 mg/L Cd	96 h	Growth inhibition Decreased cell density in culture	Monteiro et al. (2011)

(continued)

Table 4 (continued)

Model used	Environmental toxicant	Dose used	Exposure period	Toxicological effects	References
Freshwater algae (*Chlorella vulgaris*)	Ibuprofen, ciprofloxacin, and chlorophenols	20–320 mg/L ciprofloxacin, 35–320 mg/L ibuprofen, 7.5–120 mg/L chlorophenols	96 h	Inhibition of algal growth	Geiger et al. (2016)
Marine microalgae (*Skeletonema costatum*)	Polyvinyl chloride (PVC) microplastics	1, 5, 10, and 50 mg/L	24, 48, 72, and 96 h	Inhibit algal growth Decrease in chlorophyll content Alteration in photosynthesis ability of algae	Zhang et al. (2017)
Freshwater algae (*Chlorella sorokiniana*)	PS-MPs	60 mg/L	4 weeks	Decreased cell size Increased lipid accumulation Altered chloroplast galactolipids composition	Guschina et al. (2020)
Unicellular green algae (*Chlorella sp.*)	Carbon nanotubes	5, 10, 20, 50, and 100 mg/L	96 h	Inhibition of algal growth Increase ROS level and oxidative stress	Long et al. (2012)

binary and multiple fission, so the effects of environmental stressors on the regulation of cell cycle can be studied effortlessly (Bišová and Zachleder 2014). Culturing methods in laboratory conditions are also very simple as compared to mammalian cells. Due to the easy availability and fast growth of algae, ecotoxicological studies are repeatable as well as reliable. Maintenance costs are also very low as compared to mammalian cells (Silva et al. 2009). Most algae are photoautotrophic, and the effects of different environmental stressors like heavy metals and microplastics on chlorophyll content, photosynthetic capacity, oxygen consumption, and evaluation can be studied using an algal model (Neethu et al. 2021; Han et al. 2020). This will give us a complete idea of the effects of these stressors on higher vascular plants. But using mammalian cells, we cannot predict the effects of environmental stressors on plants or in other simple organisms. Using algal models, the effects of environmental stressors on cell growth parameters like cell density and cell viability can also be checked (Monteiro et al. 2011; Huang et al. 2016), as well as cytotoxic effects like oxidative stress and DNA methylation patterns can be easily evaluated (Kumar et al. 2012).

The major disadvantages of using algal models in ecotoxicological evaluation of environmental stressors are that they have very simple cellular organization and are oversensitive to environmental toxins. They are not related to humans, and we cannot predict the effects of environmental stressors on human beings and other animals by using an algal model. Very less genomic data of algae are available as compared to that of mammalian cells. Mammalian cell lines can be used to investigate the toxic effects of the toxicants on the organs of animals like hepatotoxicity, renal toxicity, neurotoxicity, cytogenotoxicity, as well as immunotoxicity in a cellular and tissue-specific manner, but cannot be studied using an algal model. For all these drawbacks of algae and advancements of mammalian cell lines, we can rely on *in vitro* mammalian cells for the ecotoxicological evaluation of environmental stressors.

7 Conclusions

All ecotoxicological models are efficient enough to give information about the toxicological potential of any environmental stressors on different perspectives. Algae are the simpler model for ecotoxicity screening of different stressors. Changes in algal density are great indicators for aquatic pollutants. Algae have very simple and short life cycles. Maintenance of algal models in laboratory conditions is also easy and cost effective. Trophic transfer of toxins and its effects on cellular level can be evaluated using an algal model. But we cannot predict the toxic effects of stressors on organism levels or higher organisms using an algal model, for which we have to use complex organisms as models. The fish model is ideal for *in vivo* toxicity screening using aquatic toxicants. The fish model has faster and shorter life cycle, and it is much easier to maintain in laboratory conditions in small spaces. Fish are easily available and have complex body systems like mammals. Using the fish model, toxicological effects on complete body system like immunotoxicity,

cytotoxicity, genotoxicity, and reproductive toxicity can be understood. Using fish models, multigenerational effects of environmental stressors can be evaluated in less time. Despite these advantages, we cannot understand or predict the effects of any environmental stressors on humans by using fish as a model, as there are many differences, like fish are mostly oviparous and external fertilization of eggs occurs in fish. Fish are also much more sensitive to stressors than humans and higher mammals. To understand or predict the effects on the human body, we have to use mammalian animal models due to their greater similarity with humans. The effects of toxins are mostly species specific, so we cannot consider the exact effects of any toxins on any species by testing on other species as models. Maintaining mammalian models in a laboratory is costly and needs a huge amount of money, and has many ethical constraints. For all these limitations, we can rely on mammalian cell lines as a model for ecotoxicological evaluation of environmental stressors. We can test *in vitro* in the laboratory using cell lines of the organs and animals of interest, including human, and understand the toxicity of any stressors. But for proper and complete ecotoxicological evaluation of any environmental stressor, multiple models from multiple trophic levels should be used beside mammalian cell lines.

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Bioimaging: Usefulness in Modern Day Research

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Contents

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_23-1

Abstract

Bioimaging involves the imaging of cells, tissues, organs, the whole body, and various cellular processes in real time. These techniques drive as little invasion as possible with cellular processes. In the medical field, ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT) are routinely used in clinics. Contrast-enhanced CT, ultrasonography, and MRI techniques are often used to provide anatomical information and also for monitoring treatment. PET and SPECT are nuclear imaging that usually gives functional information at the molecular level such as tumor metabolism. Nanomaterials such as nanorods, nanoparticles, and nanospheres are used in cancer therapy, drug carriers, and contrast agents for imaging. Continuous advancements in designing new nanomaterials have improved the development of effective cancer therapy and medical imaging. All these bioimaging techniques contribute to developing more effective treatments and reducing cancer reappearance. Microscopy techniques have an important role in imaging the biological samples, which give information about the presence of ions, metabolites, and their dynamicity within the cells. This chapter discusses the usefulness of diverse medical imaging modalities and microscopy techniques used in modern research for the betterment of early diagnosis and prognosis of diseases, and effective cancer therapies.

Keywords

Bioimaging · Microscopy · PET · MRI · CT · Optical imaging · Diagnosis

1 Introduction

Bioimaging states the methods or techniques that are used noninvasively for biological processes in real time such as subcellular structures, cells, tissues, and multicellular organisms or biological materials that are fixed for observation without affecting cellular physiological processes such as respiration and movement of cells. Bioimaging plays an important role in determining cellular processes such as quantifying metabolites, molecules, and ions levels and measuring live interaction between molecules within cells. The bioimaging techniques are frequently used to gain 3D structure information of specimens without invasion, or as little as possible interference with the cellular processes. This allows imaging of biological processes and changes in receptor kinetics, movement of molecules, and interaction through membranes of a cell. Bioimaging provides detailed tracking of metabolites that can be used as biomarkers for the detection of various diseases and responses to

treatment. More broadly, this technique includes methods for visualization of biological samples that are fixed for observation (Hildebrandt and Gambhir 2004). Bioimaging techniques use light, fluorescence, electron, magnetic resonance, positron, and ultrasound as a source for imaging. This imaging includes a broad spectrum of techniques like microscopy, PET, CT, SPECT, MRI, etc. (Fig. 1). Ultrasonography imaging uses sound waves and is commonly used to diagnose abnormality in the abdomen, pelvic cavity, and other parts of the body. CT uses X-rays for imagining to give information about internal injuries, fractures, trauma, etc. PET and SPECT evaluate functional information about tissues and cells. MRI is also a noninvasive imaging technique used in the medical field for the diagnosis and prognosis of a disease. All these techniques enable to development of new medicines, prognosis, and diagnosis of patients. Optical imaging is a highly sensitive imaging technique which gives information in real time of molecular events, biological processes, and disease processes. Over the years, bioimaging techniques underwent explosive growth and an important tool for diagnosing disease in clinics. Optical imaging includes fluorescent and bioluminescent imaging. Advancements in bioimaging include super-resolution microscopy and two-photon excitations. Hybrid modalities such as PET/CT, PET/MRI, and SPECT/CT are used for further enhancing the bioimaging results and accuracy of tumor/tissue analysis. In the near-infrared region, light can penetrate deeply into tissues as compared to UV and visible regions. Due to low light scattering, it gives rise to an emerging branch of near-infrared fluorescence imaging. So, there is a need for the development of more sensitive, highly specific, and more targeted optical imaging techniques in cancer diagnosis (Lim et al. 2017). Organic dyes and fluorescent proteins are traditionally used for labeling of cells and cellular targets due to good biocompatibility, small size, and dispersibility of water. Fluorescent probes emerge as a powerful tool with the potential to detect and screen the pathological states of pathological processes. Optical imaging is another powerful tool to detect specific molecular and disease-associated processes. Optical imaging indirectly contributes to reducing cancer reappearance and developing more effective treatment (Lim et al. 2017). Nowadays several nanomaterial-based probes are used in optical imaging. Fluorescent nanomaterials such as gold nanoparticles, quantum dots, silicon nanoparticles, and up-conversion nanocrystals have shown an advantage over fluorescent proteins and dyes.

This chapter describes the application of various techniques used for bioimaging of cells and tissues to the whole body and helps in potential use for diagnostic, theragnostic, and pathological studies of various diseases. In the medical field, CT, PET, MRI, SPECT, mammography, etc., techniques are used for the diagnosis of disease associated with the brain, heart, bones, lungs, and detection of cancers.

2 Bioimaging Modalities Used in the Medical Research

Medical imaging refers to techniques in which several images of body parts are created for diagnosis and purposes of treatment. Medical imaging includes a noninvasive test to diagnose diseases and wounds/injuries. These modalities also include

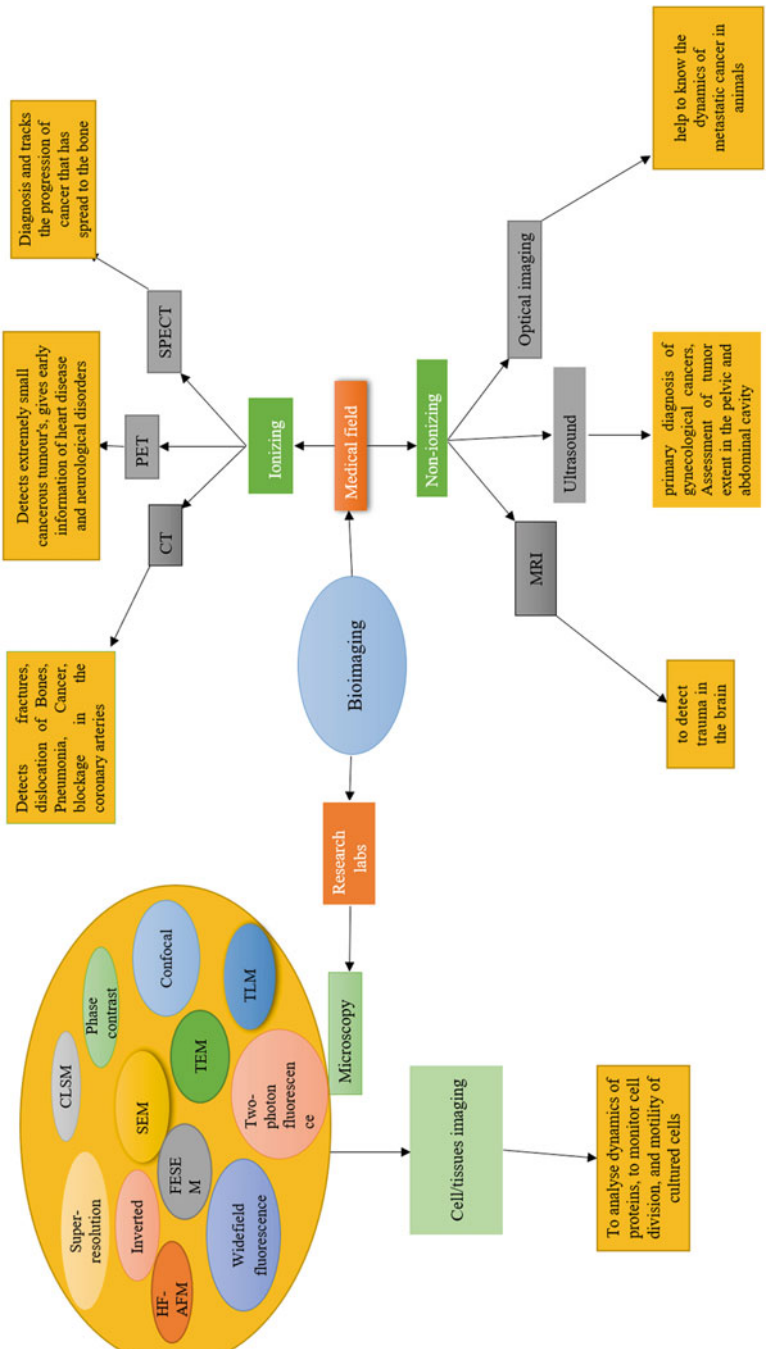

Fig. 1 Common bioimaging modalities role in clinical and research. *MRI* magnetic resonance imaging, *PET* positron emission tomography, *CT* computed tomography, *SPECT* single-photon emission computerized tomography, *TLM* time-lapse microscopy, *CLSM* confocal laser scanning microscope, *HS-AFM* high-speed atomic force microscopy, *FESEM* field emission scanning electron microscopy, *SEM* scanning electron microscopy, *TEM* transmission electron microscopy

nuclear imaging techniques such as PET and SPECT. After imaging, scans also use to see how well is the body responding to treatment for diseases and fractures. Techniques that belong to the area of radiography, i.e., X-Ray and CT scans, are very important tool in imaging, but due to ionizing radiation, their high risk of cancer, abnormal growth in the fetus, and cellular mutations, they need to be carefully used. However, MRI uses a magnetic field and reduces the risk of ionizing radiation. Ultrasound imaging uses ultrasonic vibration to create images and indicates as one of the safest techniques of medical imaging. Bioimaging techniques are PET, CT, MRI, SPECT, and ultrasound noninvasive imaging modalities are used for both human and small animal imaging (Table 1).

2.1 Computed Tomography

Computed tomography **(**CT) is a useful medical imaging technique developed in the 1970s. CT is most prominently used in diagnostic medicines. In 1979, Godfrey N. Hounsfield and Allan M. Cormack were awarded the Nobel Prize for the discovery of CT. A CT scan is a more advanced X-ray machine used to carefully diagnose the injuries, dislocation of bones, internal organs, and detection of pneumonia and cancer. This scan combines a succession of X-ray images from diverse angles of the body parts and computer processing to produce cross-sectional 3D images of blood vessels, bones, tissues, and other parts of the body. Computerized X-ray imaging is based on the principle when X-ray passed through the soft tissues and dense bones, differences in tissue density can be visualized by calculation of attenuation coefficient. It is required to use contrast agents to enhance the contrast of the diseased tissues with the use of X-ray contrasting agents, which increase the contrast between diseased tissues and normal tissues, but these contrasting agents have some limitations, i.e., nonspecific vascular permeation and due to rapid renal clearance, having a short imaging time. X-ray use in CT scan can damage the DNA of cell which can be a reason for the increased risk of cancer. These images give more detailed information than normal X-rays and are mainly used to examine internal injuries and trauma, diagnose muscle and bone disorders, and highlight the points of infection, tumors, and internal bleeding. CT detects several diseased conditions such as cancer, heart diseases, liver masses, lung nodules, staging of lymphoma but needs to carefully analyze CT scan results to get the true extent of diseases (Ritman 2004). In some cases, contrast materials can cause allergic reactions (Ritman 2004). A CT scan is a great diagnostic tool for heart disease and can detect blockage in the arteries (Swensen et al. 2003). Severe acute respiratory syndrome, SARS-CoV-2 causes acute respiratory infectious disease in humans known as COVID-19. The RT-PCR method is applied for the diagnosis of COVID-19 patients, but many of them may not be recognized at the initial appearance and have a higher chance of infecting more people and also not give details regarding the severity of the disease. A noninvasive chest CT scan is used as a fast diagnosis of COVID-19, and it also assesses the disease severity with high accuracy (Bollineni et al. 2021).

Table 1 Bioimaging modalities used in human and small animals: clinics and research

Modalities for bioimaging	Source of imaging	Contrast agents	Usefulness/advantages	Limitations	References
Computed tomography imaging	X-ray	Gadolinium, iodine, and Ba sulfate-based compounds	Used to examine the internal injuries and trauma, diagnose muscle and bone disorders, highlight the points of an infection, tumor, and internal bleeding	Less sensitivity for soft tissues	Ritman (2004)
Ultrasound imaging	Sound waves	Microbubbles, e.g., Sonazoid and Sonovue	Role in breast cancer detection, image-guided biopsy, detection of tumor extent in the pelvic and abdominal cavity, and lymph node diagnosis	Poor image quality in obesity patients and less consistent localization of visceral injury	Le et al. (2020)
MRI imaging	Magnetic field and radiofrequency	Magnetizable constituents: Fe_3O_4, Gd chelates	To know structural and functional information of the brain, to detect trauma in the brain, also used to evaluate back injuries	Image can be distorted by metals, strong, magnetic field of active implants such as cardiac pacemakers	Penet et al. (2010)
PET imaging	Gamma rays	Positron-emitting radioisotopes; ^{18}F, ^{11}C, ^{13}N, ^{15}O, ^{64}Cu	Information about whether a tumor is benign or malignant, measure body functions such as glucose metabolism, blood flow, and oxygen use by cells and tissues	Need for radiations, low-resolution images, expensive technique and does not give any anatomical information of the body	Vaquero and Kinahan (2015)
Single-photon emission computed tomography (SPECT)	Gamma rays	Gamma emitting radioisotope; I^{123}, technetium-^{99m}	Primarily used in cardiology (myocardial stress) imaging, lesions visualized by functional imaging can be correlated with anatomic structures, to guide for biopsies of bone lesions	Limited resolution, difficulty in interpreting the results, less clinical value, and not as much growth in clinical research	Jacene et al. (2008)

Optical imaging	Visible light	Fluorescent dye, IR dyes, and reporter genes	Direct imaging of the tumor for accurate diagnosis of cancer in vivo	Not yet available in clinics, low depth penetration, low resolution	Pirovano et al. (2020)
Hybrid bioimaging modalities					
PET/CT	X-ray and gamma rays	Radioisotope; ^{18}F-FDG	Used to know anatomical and functional information, PET/CT images have the potential to guide for biopsy of the tumor	Inability of simultaneous acquisition, reduced soft-tissue contrast, and sequential imaging design	Anand et al. (2009)
PET/MRI	Gamma rays and radiofrequency	Gd-DOTA-4AMP-F	Combining structural information with functional image could produce good soft-tissue contrast, high probability of detecting low-grade lymphomas, low dosage of radiations	High initial cost, limited flexibility of combined PET/MR systems, high acquisition times of up to 60 min	Musafargani et al. (2018)
SPECT/CT	Gamma rays and X-rays	Uses gamma-emitting radioisotopes, e.g., I^{123} and Tc^{99m} (a metastable isomer of technetium-99)	Used to direct correlation of anatomic information and functional information, lesions visualized by functional imaging can be correlated with anatomic structures, to guide for biopsies of bone lesions	Less clinical value and not as much growth in clinical research	Israel et al. (2019)

2.2 Ultrasound Imaging (Ultrasonography)

Ultrasound (US) is used as a diagnostic imaging technique in the medical field. In 1956, it was the first time use for clinical reasons by Ian Donald and Tom Brown. Ultrasound machines form images by release high-frequency sound waves through the patient body. As the sound waves bounce off tissues and organs, they create echoes and are made into pictures by a machine. Ultrasound imaging shows the structure and movement of organs and is also used for imaging blood flow through blood vessels. Soft tissue disease pictures are also obtained from the ultrasound that are not shown by the X-ray. A fluid-filled cyst from solid tissues can be differentiated due to the formation of different echo patterns. Doppler flow ultrasound machines are used to display how fast and in which direction blood flows in vessels. Ultrasound is a noninvasive, normally accessible, inexpensive imaging method with negligible risk to the patient. Ultrasound imaging shows an important role in the diagnosis of gynecological cancers (cervical cancer, uterine cancer, vaginal cancer, etc.), evaluation of tumor degree in the abdominal and pelvic cavity, and the assessment of the effects of treatment. It also allows the targeted biopsy of metastatic lesions or advanced tumors (Fischerova 2011). Ultrasound limitation includes its dependency on operator training and skill, a low-quality image in obese patients, and less consistent localization of visceral injury as compared to CT. Efficacious use of ultrasound can be maximized with some factors like suitable training of sonographers and improvement of technical limitations.

US imaging has a significant role in the diagnosis of lymph nodes, image-guided biopsy, and detection of breast cancer (Guo et al. 2018). Ultrasound can evaluate the internal structure, orientation, morphology, and margins of lesions with high resolution in glandular structures and fatty breasts. This imaging is also used to differentiate between benign and solid lesions (Le et al. 2020). Several preclinical studies demonstrate the high potential of targeted ultrasound imaging for improved characterization of tumors and to monitor treatment response with high sensitivity**.** Ultrasound molecular imaging has also been developed as a promising tool for the visualization of tumor angiogenesis. However, ultrasound images are not good as MRI and CT and are also limited to a particular part because the sound waves do not pass through the air in the lungs and bones.

2.3 Magnetic Resonance Imaging (MRI)

In medical research for diagnosis, MRI is a noninvasive imaging technique that provides a better chance to avoid exposure to dangerous ionizing radiations. The less cost and better availability make it more persistent throughout clinical research. Nuclear magnetic resonance phenomena were first described by Shampo et al. (2012), and the technique evolved as the use of nonionizing electromagnetic fields that allow the development of clinical applications. In Mallard (2006), produced the first clinical images and now MRI is widely applicable in clinical research. MRI has advanced in many years being a good technique with prodigious potential for the

primary diagnostic investigations of many clinical problems. MRI offers detailed information on the function of organs and the internal anatomy of the body in disease and health. MRI uses three different frequencies: estimate-varying gradient magnetic field, static magnetic field, and pulsed radiofrequency fields. This is used as a primary diagnostic tool for many clinical problems. Although MRI can provide detailed information about the patient's condition, the results may be distorted by metals and strong magnetic fields. MRI is difficult to use in such cases. Moreover, patients could develop an allergy to the contrasting agents. MRI is unable to distinguish between benign and malignant tumors. Air, teeth, bones, and metallic objects all appear black, so it makes it difficult to differentiate between them. The progress of molecular targeted contrast agents has extended the old strengths of magnetic resonance spectroscopy (MRS) and MRI for describing the functional parameters of tumors such as metabolism, vascularization, pH, and cell death, and for visualizing molecular pathways. MR imaging is well appropriate to measure cellular and molecular processes including apoptosis, metabolism, cell proliferation, and biosynthetic pathways of metabolites in vivo in various tissues including cancers (Penet et al. 2010). This was initially restricted to the neuro-axis but later covered all parts of the body. Indeed, MRI is now accepted as a corresponding technique on parity with ultrasound and mammography (Penet et al. 2010).

2.4 Nuclear Imaging: PET and SPECT

Nuclear imaging creates images by detecting radiation from body parts after injecting a radioactive tracer. This imaging technique produces images of the spatial distribution of radioactive materials introduced inside the body instead of producing images of the anatomical maps in the support of other medical imaging techniques X-ray, MRI, and ultrasound. Nuclear medicine imaging recognizes disease biochemical indicators by imaging clearance and biodistribution of radiotracers entering the body. An increase or decrease in the rate of radiopharmaceutical accumulation in specific tissues is a strong indicator of disease. And images are generated on a computer that is interpreted by nuclear medicine physicians to make a diagnosis and plan for treatment for the body parts being assessed.

2.5 Positron Emission Tomography (PET)

PET is used to image metabolic processes, physiological activities, and molecular targets in the body. Positron emission tomography based on recognition of two time-coincident high-energy photons from radioisotope emits a positron. The first-time PET was device used with two opposed sodium iodide detectors for imaging by physicist Gordon Brownell and neurosurgeon William sweet to detect brain tumors. After the success of the prototype of PET in the 1950s, the first clinical positron imaging device was designed in 1952, particularly for brain imaging, with many refinements in the scanner. The first PET camera for human studies was built by

Michael E. Phelps, Edward Hoffman, and Michael M. Ter-pogossian (1975). Initially, PET imaging was based on the ^{15}O labeling to O_2, CO_2, and CO largely. ^{15}O remained a very useful label for PET imaging for studies of blood flow in the brain and other organs. Labeled CO_2 was used to obtain equilibrium images of the blood bow of the brain and heart of animals and humans, and labeled CO was used to measure regional blood volume (Brownell and Cochavi 1978). ^{18}F labeled, 2-fluorodeoxy-D-glucose (2FDG) was developed as a major factor in increasing the opportunity of PET imaging, because the ^{18}F half-life is 110 min which is optimal for PET imaging and facilitates greater availability for PET imaging. FDG is an analog of glucose and tends to accumulate in tissues with higher glucose demands. It appears that the slow metabolism of glucose in malignant tumors due to warburg effect, can be detected in vivo by the accumulation of FDG (Ido et al. 1977). FDG could give values of energy metabolism of the brain, heart, and other organs also (Reivich et al. 1979). PET imaging has emerged as a potential tool for diagnosis and staging of cancer and is also used in cardiovascular and neurological signals. This imaging is based on the emission of a positron from a radioisotope deficient in neutrons (Vaquero and Kinahan 2015). Nowadays, over 400 scanners are used worldwide, including PET-18FDG scans, cardiac PET scans, and PET/CT scans.

Furthermore, a major application of PET is non-invasive tumor imaging, and it can detect primary tumors and distant metastasis. Thus, a PET scan is a powerful diagnostic test that noninvasively gives information about whether the tumor is benign or malignant, and it measures body functions such as glucose metabolism, blood flow, and oxygen use by the body to evaluate the tissues, organs functioning. In brief, PET imaging can detect cancerous tumors, elusive changes of the brain and heart, and gives early information of heart disease and neurological disorders, i.e., Alzheimer's. PET scan suggests earlier treatment in the course of the disease and provides better chances of treatment.

2.6 Single-Photon Emitted Computed Tomography (SPECT)

SPECT is a type of nuclear imaging used to analyze the functions of the internal organs. A radionuclide is injected or introduced into a molecule of potential biological interest and administered to a patient/animal. A special camera is used to form 3D images. In SPECT imaging, internally distributed radiopharmaceutical penetrates emit gamma-ray photons, detected by collimated radiation detectors. SPECT imaging study is helpful in thyroid imaging, infection imaging, tumor imaging, and also provides information and functioning of organs. Various drugs and chemicals are labeled with radioisotopes for SPECT imaging. Radiotracer used in SPECT scan may affect developing fetus (Jacene et al. 2008). SPECT scan is frequently used to diagnose hidden bone fractures. Also, it is used to diagnose and track the progression of cancer that has spread to the bones. SPECT scans help in determining the affected parts of the brain by head injuries, dementia, epilepsy, and clogged blood vessels.

3 Hybrid Modality

3.1 PET/CT

PET/CT performs most of the PET scans and provides greater detail with a more precise diagnosis than the two types of scans performed distinctly. It provides greater convenience for the patient who undergoes both tests (PET and CT) at one time. One limitation of the PET/CT is the radiotracer that can take many hours to days for accumulation in the area of interest. The radiotracer decay time is quick and is effective for a short time. Major suppressing in PET/CT is the sequential imaging from two systems that can cause errors due to patient movements between two procurements (Anand et al. 2009). Whole-body ^{18}F-PET/CT has been established as a significant imaging type for the diagnosis, staging, tracking of the treatment response, and assessment of the long-term valuation in breast cancer patients.

3.2 PET/MRI Scanner

Integration of PET/MRI scanners was the first effective combination of two modalities allowing simultaneous achievement of their imaging signals. Integration of these, resulting from the development of precise hardware, paved the way for new methods to study neurodegenerative disorders. Hybrid PET/CT and PET/MR imaging modalities enhance the analysis of brain tumors. PET/CT reduced contrast in soft tissues led to the development of PET/MRI which gives a result in the combination of structural data with functional images. PET/MRI was introduced in 1990 with the suggestions of PET/CT but not as successful as PET/CT due to various challenges in hybrid modality. PET/MRI uses lower exposure of radiation, has a higher contrast of soft tissue than PET/CT, and can be used on any part of the body. A low dose of radiation is also helpful for imaging in small animals. PET/MRI goes out to be potent in many preclinical and clinical applications. This technique has gross application in oncology, neurosciences, and musculoskeletal tissues. Future investigations also require on PET/MRI for clinical and preclinical trials. PET/MRI is costly than PET/CT but performs a high possibility of detecting low-grade lymphomas (Musafargani et al. 2018). New clinical indications develop for hybrid imaging with PET/CT or PET/MRI.

3.3 SPECT/CT

SPECT/CT scan is an integration of SPECT and CT scan. By integrating both these techniques, we can show a detailed and informative study about anatomy and physiology. It helps in the early-stage detection of disease before other imaging tests. Neither CT nor SPECT alone can recognize the locus of malignancy, but a hybrid SPECT/CT image helps the surgeon precisely recognize the site where to operate (Israel et al. 2019).

In brief, bioimaging is an influential tool to look at the internal workings of the human body and diseases. All the bioimaging techniques like CT, ultrasound, MRI, SPECT, and PET have a role in the diagnosis of several diseases and treatment at an early stage. There is a need for more advances in these techniques to diagnose the disease at an early stage. Mammography is used for breast imaging by taking X-ray pictures of the breast for diagnosis and screening. It is used to check breast cancer when no sign of disease or an early sign of breast cancer (Gøtzsche and Jørgensen 2013). Breast MRI is used for knowing the extent of breast cancer and helps in planning for treatment. Breast ultrasound helps in differentiating fluid-filled cysts, cancerous tumors, and benign tumors, and there are some studies of PET/CT mammography which show the better utility of ^{18}F-FDG as compared to MRI mammography. FDG-PET/MRI mammography is done for breast imaging in addition to PET/MRI scan for breast cancer patients without the addition of radiation and can be an effective imaging technique in breast cancer patients (Cho and Kong 2017).

4 In Vivo Optical Imaging Method

Optical imaging is fast, cost-effective, accessible, and helps in the study of noninvasive molecular and biological processes of diseases, and the development of new drugs using fluorescent and bioluminescent probes. As given in Table 2, bioluminescent imaging (BLI), fluorescent imaging (FLI), and Cerenkov luminescence imaging (CLI) offer exclusive features for imaging small animals (Ciarrocchi and Belcari 2017). BLI is used to monitor tumor growth in deep tissues with high specificity, more sensitivity, and low background signal from tissues (Tung et al. 2016). FLI use is perfect for quantifying and monitoring cell behavior, and FLI use is perfect for quantifying and monitoring cell behavior (Hoffman 2015). Cerenkov luminescence (CLI) allows the visualization of light emission in mice from commonly used, clinically approved radiotracers. When a charged particle passes through a medium faster than the speed of light, photons are emitted, and this is a phenomenon of Cerenkov radiation. Visible light was detected due to the Cerenkov effect in laboratory animals like rats after radionuclide administration. The CLI method allows the spatial distribution of biomolecules labeled with radionuclides (Das et al. 2014). Optical imaging techniques such as bioluminescence and fluorescence imaging are low-cost, vigorous, and more sensitive methods, particularly bioluminescence, because they give minimum background signal from tissues. But optical imaging is primarily applied to small animals in the laboratory and is not usually used for human and whole-body imaging because of limited depth sensitivity and low spatial resolution. Optical imaging has an important influence on the surface examination of targeted regions like carotids, lymph nodes, and exposed surgical areas. Cells need to be genetically modified for bioluminescence imaging so that they express the luciferase gene activity and administer the substrate to the host animal. This is the most sensitive method to track cells, tumor growth, metastasis spread, and antitumor treatments in in vivo. For fluorescent imaging, it needs to

Table 2 Optical imaging methods used in vivo study in small animals

Methods of optical imaging	Principle	Advantages	Limitations	References
Bioluminescence imaging (BLI)	Light emission on oxidation of substrate by a luciferase	Used for tracking of cancer cells, a signal is more sensitive, no need for excitation light so no risk of photobleaching and phototoxicity, and suitable for light-sensitive cells, e.g., retinal neurons	Limited temporal resolution	Tung et al. (2016)
Fluorescence imaging (FLI)	Absorption of light by fluorescent dyes/proteins and emission of light after relaxation	Enabled the dynamics of metastatic cancer to be followed in real time in individual animals, less sensitive with higher background signal	Can cause photobleaching and phototoxicity	Hoffman (2015)
Cerenkov luminescence imaging (CLI)	Light emits when high-energy charged particle (positron) passes through a medium	In vivo tumor imaging	Needs radioactive reinjection	Das et al. (2014)

express fluorescent protein by cells or administer fluorescent dye by the cells. The technique is helpful in monitoring tumor, ion tracking, and metastasis spread. The technique is also used for molecular imaging of biomarkers for cancer, bone metabolism, inflammation, and apoptosis (Pirovano et al. 2020). Photoacoustic imaging is a noninvasive, emerging type of imaging that combines deep optical contrast and high penetration of ultrasound. PAI uses nonionizing laser pulses to deliver energy to the tissue for the emission of ultrasonic waves. It gives better resolution than only optical imaging and deeper penetration than only ultrasound imaging. It is an emerging diagnostic modality that gets the benefit of ultrasound signals induced optically. Photoacoustic imaging is clinically used for imaging of breast, skin, and gastrointestinal tissue (Attia et al. 2019).

5 Bioimaging at the Cellular Level Using Microscopy

Microscopy for a long time was used for visualization of the biological structures and processes within the cells, tissues, and organisms. Bioimaging plays important role in the cellular level in life sciences research. And it is used to analyze molecules,

cells, and tissues. Development in microscopy techniques makes it easy to allow imaging across a wide range of 1–2 nm. The first microscope was invented by Zaccharias Janssen in about 1590. All living matter is active therefore need to monitor the dynamics of living systems over multiple lengths and time scales over the single cell to the whole organism. It is important for the understanding of the inner functioning of the human body. Live-cell imagers and microscopes use time-lapse photography to visualize live cells and do not require severe light and toxic dyes. Some microscopy techniques that can capture live cell images are fluorescence microscopy, confocal microscopy, and quantitative phase-contrast microscopy. Some microscopy techniques are discussed in Table 3.

5.1 Inverted Microscopy

Inverted microscopes are used for the observation of living cells or organisms in tissue culture in more natural environments than using conventional microscopes (Ramadurai et al. 2020).

5.2 Differential Interference Contrast Microscopy

Differential interference contrast microscope commonly uses the light near-infrared region for visualizing unstained samples. Also, it is used to imaging thick specimens, i.e., brain slices, and imaging unstained live cells (Wang and Fang 2012).

5.3 Phase-Contrast Microscopy

Phase-contrast microscopy itself was a great achievement in the field of microscopy. The principle of phase-contrast was given by Zernike (1935) and a Noble Prize was awarded for this in 1932. This technique is used in cell culture and allows living cells to be observed in natural conditions without fixation. Cells without stain practically do not absorb any light, so small intensity changes in the image due to poor light absorption. In a phase-contrast microscope, phase changes induced due to specimen parts' thickness and refractive index can be transformed into image contrast (Barer 1947).

5.4 Fluorescence Microscopy

Fluorescence microscopy is the type of light microscope that works on the principle based on fluorescence. Before observing, the sample should be labeled with fluorescent dye or substances. The most common type of fluorescence microscope is the

Table 3 Microscopy techniques used in cell/tissue imaging

Microscopy	Cells/tissue	Advantages/ applications	Limitation	References
Inverted microscopy	Live cell imaging	Observing living cells or organisms in a tissue culture flask under more natural conditions	Limited magnification	Ramadurai et al. (2020)
Differential interference contrast microscopy	Single-cell organisms/ tissues	Visualizing unstained samples, imaging thick specimens such as brain slices	Not ideal for thick organisms or particles	Wang and Fang (2012)
Phase-contrast microscopy	Live cell imaging	Living cells can be observed in their natural state without labeling and staining, diagnosis of tumor cells	Sometimes difficult to differentiate between cells undergoing mitosis and early stages of apoptosis, not ideal for thick organisms or particles	Barer (1947)
Widefield fluorescence microscopy	Entire specimen or used for specific protein or component of cell	Detection of specific structures, molecules, or proteins within a cell	Not suitable for the 3D image and difficult to tell how deep fluorescence came out from tissues	Cardin et al. (2020)
Two-photon fluorescence excitation microscopy	Live cell imaging of thick biological specimens	Less photobleaching and photodamage, provide large depth penetration	Use of high-intensity light can destroy cell	Diaspro et al. (2005)
CLSM (confocal laser scanning microscope)	Cells also use in vivo imaging of human epidermis and superficial dermis	Enabling access to dynamic cellular and molecular processes, allowing early diagnosis and high-resolution images	High cost and relatively smaller field of vision	Bayguinov et al. (2018)
High-speed atomic force microscopy (HS-AFM)	Proteins and live-cell imaging	To analyze dynamics of proteins, to visualize dynamic behavior of biomolecule	Cannot visualize small molecule that interacts with proteins	Miller et al. (2018)
Scanning electron microscopy (SEM)	Bacteria/cells	Observe small structures on the surface of cells	Cannot use to see live specimens/cells	Erlandsen et al. (2000)
Transmission electron microscopy (TEM)	Section of tissues and cells	Give information of the inner structures such as morphology and stress state information	Samples must be very thin (normally $<$150 nm)	Mielańczyk et al. (2015)

(continued)

Table 3 (continued)

Microscopy	Cells/tissue	Advantages/ applications	Limitation	References
Time-lapse microscopy (TLM)	Living cells imaging	Use for comparative studies of cultured cells, to monitor cell division, cell migration, and motility of cultured cells	More clinical research is required to prove that TLM can identify the best embryo transfer	Baker (2010) and Svensson et al. (2018)
Super-resolution microscopy (SIM, STED, PALM, etc.)	Live-cell imaging	Used for cellular components in the range of 10–200 nm and breaks all the diffraction barriers of conventional microscopy	Difficult to implement and poor performance in thick specimens (e.g., embryos, tissues)	Georgieva and Nöllmann (2015)

widefield fluorescence microscope. Widefield fluorescence microscopy is the easiest mode of fluorescence imaging used for the detection of proteins, specific structures, and molecules present in cells. In widefield microscopy, the whole specimen is exposed to light (Cardin et al. 2020). Two-photon fluorescence excitation microscopy is also a type of fluorescence microscopy. Two-photon fluorescence excitation microscopy is used for live-cell imaging of thick biological specimens. The use of this microscopy leads to less photobleaching and photodamage, providing large depth penetration of the sample (Diaspro et al. 2005). Total internal reflection fluorescence (TIRF) microscopy is also used for live-cell imaging. It uses total internal reflection to produce high contrast and low background images. TIRF microscopes are used for applications as single-cell analysis, surface receptor activity, cell membrane study, cytoskeleton dynamics, and other live-cell imaging needs (Fish 2009). FRAP is a method used in fluorescence microscopy for studying the fluorescently labeled molecules in living cells, and the FRET technique is applied for visualization of protein-protein interactions and changes in cellular calcium concentration using widefield fluorescence microscopy.

5.5 Confocal Laser Scanning Microscopy (CLSM)

Confocal microscopy is a fluorescence imaging technique that produces optical sections by passing a laser over the sample and collecting the data and form a final image. It is useful in studying live-cell imaging. The basic types of confocal microscopy are spinning disk microscopes, confocal laser scanning microscopes, and programmable array microscopes. These types of microscopes are found in the research laboratory and biomedical research at demand due to high sensitivity. Two main categories of CLSM are single point used for slice imaging and array scanning used for live-cell imaging. CLSM is an advancement in light microscopy that

explores the latest laser, imaging, and computational techniques to enable biologists to visualize the cell and subcellular components in distinctive forms (Minker et al. 2018). Confocal microscopy with Ca^{2+} sensitive fluorophores used in the analysis of Ca^{2+} dynamics in ventricular or atrial cardiomyocytes is also used in intact cells without any interference in structural and functional mechanisms. CLSM (confocal laser scanning microscopy) is used to see visual sections of small structures such as embryos and form a 3D structure image using a laser beam that scans point by point of the object (Bayguinov et al. 2018). CLSM is an ideal technique for host-pathogen interactions and studying the developmental processes of the pathogens with host tissues.

5.6 Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

SEM and TEM are electron microscope techniques. In SEM, images are produced by focusing the electron beam on the sample. In TEM, an electron beam is passed through the ultrathin specimen, interacting with the specimen gives Information of the inner structures such as morphology and stress state of the specimen (Mielańczyk et al. 2015). The arena of SEM has undergone a revolution in the last decades. In the late 1980s, advancement in field emission scanning electron microscopy **(**FESEM) with the lens collection for secondary electrons gives advanced resolution in the nm range possible. The development of cryo-SEM led to an interpretable structure of about 1–2 nm on biological samples. FESEM produces less distorted, clearer, and three to six times better images compared to SEM. Organisms that are responsible for endometritis and infertility in mares were achieved with the help of FESEM to observe three different phases of *Streptococcus zooepidemicus*, which is hidden for a prolonged time in the endometritis and causes infertility in mares, and enabling it to hide from the immune system (Erlandsen et al. 2000).

5.7 High-Speed Atomic Force Microscopy (HS-AFM) in Bioimaging

HS-AFM is an exclusive technique used for direct visualization of interactions of proteins and structural changes deprived of labeling. The first time was stated in 2001, and since then it is used to study the dynamics of proteins, motor proteins, DNA-binding proteins, artificial proteins, and amyloid proteins. Advancement in optical microscopy allowed to track movements of proteins at the molecular level but the images are not of themselves proteins but regions of fluorescent intensity emitted by the fluorescent molecules bound to the protein (Miller et al. 2018). So, in these observations, only proteins' behavior is indirectly determined. So, to understand the behavior of the protein naturally, AFM is used which is part of scanning probe microscopy. The key feature of this technique was independent of the environment and applied to the application of biological samples from nucleic acids, proteins, and

chromosomes (Miller et al. 2018). The development of HS-AFM imaging was used for intermolecular interactions of proteins and conformational changes. Currently, HS-AFM is the only technique that enables to visualization of the dynamic behavior of biomolecules in a solution in real time at a nanometer scale. Some technical difficulties, i.e., disturbances are caused due to direct contact of the probes with proteins and influence their physiological functions and structures, are not negligible, so further improvements in technical development are required. HS-AFM is applied in most cases to isolated and purified proteins, and not to complex molecular systems. Furthermore, HS-AFM can not be used to visualize small molecules that interact with encapsulated protein molecules. A combination of HS-AFM and optical microscopy is used for complicated molecular systems (Umakoshi et al. 2020).

5.8 Time-Lapse Contact Microscopy Application

Time-lapse microscopy (TLM) is a powerful microscopy technique used for living cell imaging in real time. And originally, it was known as time-lapse cinemicrography. For visualization in the light microscope, cells are fixed and stained, and that process kills the cells, but with the introduction of the phase-contrast microscope in the 1940s, fluorescence, multidimensional microscopy, and flow cytometry discoveries made live cell imaging a common approach. This is also the progression of TLM to use for biological processes. Difficulties arise from old methods of observing tissues by fixation, embedding, sectioning, and staining. Working with dying tissues makes researchers think about observing living tissues. From the past years, great progress makes discoveries of phase-contrast microscopy, tissue culture methods, and time-lapse cinephotomicrography. Combining these three methods formed a research method called phase-contrast time-lapse cinephotomicrography of living cells and tissues. The principle of time-lapse cinematography is based on the production of silent motion pictures at a lower frequency than the frequency used to play the sequences of pictures. This technique modifies the duration of events occurring in a particular process. The sequence of events is observed at a faster rate than their actual occurrence. Hence, this technique is the manipulation of the apparent duration of any event by changing the speed at which it is recorded. The first time in 1904, Bull and Pizon recorded bacterial growth by this technique. In modern times, TLM is used for observing and studying cellular processes, cell-cell interactions, cellular dynamics, and behavior of living cells in a population of cells (Baker 2010; Svensson et al. 2018). TLM is also efficiently used for studying prokaryotic and unicellular organisms used for comparative studies of cultured cells, monitoring cell division, living blood, and lymph cells, defining the cytotoxic effects of allografts. TLM is also used for monitoring cell migration and motility of cultured cells, and it also helps in monitoring the distance of migrating cells and counting the number of migrated cells. TLM is used to study mesenchymal stem cells, hematopoietic progenitor cells, primordial germ cells, microglial cells, neurons, migration of osteoclasts to the sides of bone surfaces, and movement of

endothelial cells for studying the remodeling of intercellular junctions. TLM is also used to visualize cell-cell contact, the interaction between various kinds of stem cells and the tumor microenvironment, the interaction between brain tumor and microglia, and the intracellular dynamics of subcellular organelles of cells. TLM helps in monitoring changes and movement in mitochondria, centrosomes, microtubules, and Golgi apparatus. Also, TLM is used for multicellular organisms, viruses, and visualization of bacterial colony morphologies. TLM is a promising technique in clinical for selecting embryos for transplantation and is also used for the analysis of sperm motility. But more clinical research is required to prove that TLM can recognize the best embryo transfer as compared to the conventional method of embryo implantation. TLM can also use as the diagnostic for detection of anomalies in the behavior of cultured dystrophic cells, estimating tumor malignancy, and evaluating side effects of drugs such as antibiotics. TLM is also used for understanding various conditions such as thrombus formation, erythroblast development from patients with dyserythropoietic anemia, reprogramming of pluripotent cells after induction, and other applications.

5.9 Super-Resolution Microscopy

Super-resolution microscopy (SRM) makes it possible to visualize a wide range of subcellular components with molecular-scale details. SRM is an optical technique that resolves cellular structures such as Golgi apparatus, neural synapses, and nuclear membranes beyond the diffraction limit, which can be explained at the subcellular level, which is not possible with conventional light microscopy. Electron microscopy offers a high resolution and detailed study of cellular structures, but it is unsuitable for living systems and does not provide the identity of molecule-building subcellular structures. AFM provides higher resolution than EM and is used for live imaging, but it can explore the surface structures. A super-resolution microscope acquires images with two to ten times higher resolution than light microscopy using photoactivated localization microscopy and structured illumination microscopy. SRM breaks all the diffraction barriers of light microscopy. In PALM, it utilizes fluorophores, and single-molecule images can be acquired by forcing the fluorophores to blink. STED microscopy is based on confocal microscopy and reaches the sub-diffraction resolution by decreasing the size of the detected point spread function. Light microscopy imaging innovations are SIM, STED, PALM, STORM, and FPALM. These are all included in a new class of super-resolution microscopes that are made for the use of a wide variety of imaging applications like live-cell imaging, 3D fluorescence imaging, and also in-vivo imaging. Super-resolution microscopy approaches, i.e., PALM, SIM, and STORM, were established for further improvement in resolution. Despite the great resolution in imaging that can be achieved by super-resolution microscopy, there are some limitations to the use of this technique. This includes the need for extensive hardware to overcome the spatial resolution correction. This makes the use of this technique difficult (Georgieva and Nöllmann 2015).

6 Bioimaging in Cancer Research

Cancer is the leading cause of death and great efforts are made for early detection of cancer to provide more effective cancer treatment. So, bioimaging techniques were developed to improve the accuracy of cancer detection at an early stage. The bioimaging field is huge, so here we focus on the techniques which are widely used in cancer research. Bioimaging is an important part of cancer research used in the diagnosis and treatment of various cancers. Various bioimaging techniques of fluorescence microscopy such as FRAP, FRET, and confocal microscopy are often used to study the characteristics and complexity of cancer cells. Live-cell imaging helps to differentiate cancer cells from the environment they exist. The development of various bioimaging techniques such as CT, MRI, SPECT, and PET helps in advancements in the diagnosis of cancer. Nanomaterial-based probes are used in imaging to improve the quality of research to diagnose the disease (Barman et al. 2021). Light microscopy use was predominant initially, but later after the 1960s, advancement started in fluorescence microscopy. During the 1990s, dye-based fluorescent techniques were developed for the detection of the autophagy process. Different imaging techniques, i.e., PET, CT, MRI, and optical imaging, have gone through rapid development in previous decades, and these remain the main tools in the diagnosis and staging of cancer in clinics (Vadivambal and Jayas 2015).

7 Bioimaging Role in Cellular Research

At the cellular level, bioimaging is used to examine the biochemical mechanisms, quantify metabolites and ion levels, and observe interaction with molecules inside the cells. Bioimaging gives essential information about physiology at the cellular level. At the molecular level, cells turn into self-reporting for metabolites due to the design of several nanosensors used for various ions and metabolites. Bioimaging also offers essential information about physiology at the cellular level. High-resolution bioimaging techniques provide great insight into biochemical mechanisms and cellular processes. Some techniques based on X-ray, fluorescence, electron, and other sources give a high spatial resolution of molecules inside cells and tissues. Irregularities of cholesterol dispersal at the cellular level are one of the hallmarks of various lipid storage disorders. With the help of using fluorescence probes, which specifically bind to cholesterol, provide trafficking and visualization of cholesterol. The HS-AFM technique is used for observing intermolecular interactions of proteins and the dynamics of biomolecules inside cells. The use of silver nanoparticles and imaging with the transmission electron microscope gives an idea of more detailed information about localization and cellular uptake mechanisms. Scanning helium ion microscopy (SHIM) is based on a helium ion beam, which brings the major advantage of high sensitivity and a large depth of fluid in imaging biological samples like viruses and some microorganisms at nanometer resolution (Schmidt et al. 2021). Multiplexed imaging unlocks new ways to understand the pathology and is suitable to recognize the biological signs. Confocal laser scanning

and fluorescence microscopy are also used for microstructure studies, but to visualize some specific structures needs extra staining and becomes an invasive and time-consuming process. A combination of some techniques like stimulated Raman scattering microscopy, confocal Raman microspectroscopy (CRM), and Fourier transform infrared (FTIRM) is used to understand the cell wall of fruits (Huang et al. 2020). Nowadays, in cell transplantation research, the use of bioimaging rats makes it possible to visualize the in-vivo kinetics of transplanted cells and provide effective methods for cell therapy. To study cellular targets, one needs high spatial and temporal resolution that can differentiate the target from molecular events and cellular organelles. The study of live cells is essential to understand the cellular processes. Synchrotron X-ray tomography gives bone cell network information at a nanoscale (Peyrin et al. 2012). Multimodal bioimaging techniques such as X-ray, fluorescence, and MRI are used to improve the precise diagnosis of tumors. Nanoclusters are used as a probe in multimodal imaging techniques to improve the sensitivity and specificity towards the tumor tissues.

8 Bioimaging and Nanomaterials

Recently, the uses of nanoparticles provide significant progress for cancer diagnosis and image-guided cancer therapy. Signals obtained by fluorescence nanoparticles help during surgery in distinguish between the normal tissues and tumor position by real-time guidance and increase the survival rate of patients. Nowadays, optical probes based on nanomaterials are widely used in monitoring the biological pathways for cancer diagnosis and therapy. The use of fluorescent nanomaterials such as gold nanoparticles, quantum dots, and silicon nanoparticles shows the advantage of resistance to photobleaching over fluorescent dyes and proteins. Nanomaterials after modification with diverse molecules can be delivered to the site of the tumor (Rani et al. 2019).

9 Bioimaging in Drug Discovery and Development

Recent advances in noninvasive imaging techniques offer new scenarios for the discovery and development of new therapy. CT, ultrasound, and MRI are related to structure-oriented information, but functional MRI (fMRI) is used to know functional information of the brain, i.e., brain activity. Multimodality imaging strategies are emerging in the drug discovery field, which allows high-resolution data with molecular information. MRI, optical imaging, and ultrasound detection methods are used in the medical field as valuable tools in early drug discovery and pharmacology of animals. In animal models, imaging of diseased tissues and organs plays an incredible role before and after treatment with drugs. MRI and fMRI are beneficial in characterizing the pathophysiology situations and drug effectiveness of clinical candidates in the brain (Rudin et al. 2003). Quantitative magnetic resonance is used for measuring bone mineral content, bone mineral density, and fat composition of the

whole body in mouse models (Taicher et al. 2003). Optical coherence tomography (OCT) is analogous to ultrasound in developing for cross-sectional images in that it uses back-reflected light rather than sound waves. It is used to diagnose osteoarthritis and coronary artery disease. OCT can detect early signs of osteoarthritis having loss of cartilage polarization sensitivity and disruption of bone cartilage interface (Sittampalam and Westmore 2003).

10 Image-Guided Therapy

Surgery is the keystone for the cure of many solid tumors. Image-guided surgery is increasingly superior with bioimaging techniques such as CT, MRI, PET, and ultrasonography. Medical imaging techniques by image-guided surgery allowing the tumor excision and minimization of the side effects. Open configuration MRI systems help to guide biopsies and neurosurgery. This is used to guide for biopsies of lesions. Functional imaging of MRI/PET/SPECT makes possible to map brain function area such as speech center. More than 400 hybrid PET/CT scanners installed globally are used for the diagnosis of diseases, to guide biopsies, optimize doses, and monitor chemotherapy response (Yap et al. 2004). Optical imaging is most suitable in image-guided surgery because fluorescence signals can guide the distinction between margins of positive tumors and local masses from normal tissues. But the penetration depth in tissues is limited by optical imaging.

11 Conclusions

Bioimaging is an influential tool in modern research, provides real-time observation of life processes at the molecular level, disease development pathways, and cellular pathways. Several noninvasive bioimaging techniques enable effective cancer diagnosis, image-guided therapy, and discovery of novel drug discovery. Imaging techniques with high-resolution help in great insight into understand the biochemical processes and also offer important information about the physiology of cells. Optical imaging has a significant role in the observation of molecular processes, and it enables efficient supervision of cancer both in-vivo and in-vitro with more accuracy of imaging. As described in this chapter, PET, MRI, CT, and ultrasound imaging techniques used to detect and diagnose the diseases are also used to examine the internal injuries, site of infection, internal bleeding, and trauma. At the cellular level in research labs, microscopy has an important role in detecting small molecules, ions, metabolites, and proteins. Recently, emerging techniques like confocal laser scanning microscopy, super-resolution microscopy like SIM, STED, etc., have advancements in live-cell imaging, increasing and breaking the barriers of image resolution (Georgieva and Nöllmann 2015). No one technique can be enough to know information about biological systems, and the main hurdle in bioimaging techniques is resolution and quantification. Imaging methods are used to evaluate the effectiveness of treatment and investigate the mechanism of disease. Bioimaging

modalities have a crucial role in the diagnosis of a variety of diseases, detection, surgery, and monitor drug response (Fig. 2). Bioimaging also helps the surgeon to image-guided surgery by imaging cancer location. As discussed in the chapter, PET imaging is often used in clinical research areas noninvasively to look at the pharmacokinetics, distribution of drugs, delivery of genes, cellular proliferation, and trafficking. The development of hybrid bioimaging modalities such as PET/MRI, PET/CT, and SPECT/CT have increased the accuracy of detection of disease problems and aided in understanding pathology of disease. In this chapter, we concluded that imaging methods have a potential role in the early detection and diagnosis of various diseases or abnormalities in the body. Imaging methods also give a better chance to start the treatment and recovery at an early stage due to the early detection of diseases.

12 Challenges in Bioimaging and Future Direction

Although bioimaging methods have an important role in imaging cellular signaling, biological processes, and the movement of molecules through the cell membrane, they are mostly noninvasive. Also, these methods are used for tracking ions and metabolites that can be used as biomarkers for the identification and progression of diseases. A potential challenge of bioimaging studies is quantitation, and in the case of some microscopic techniques, sample preparation is a time-consuming process.

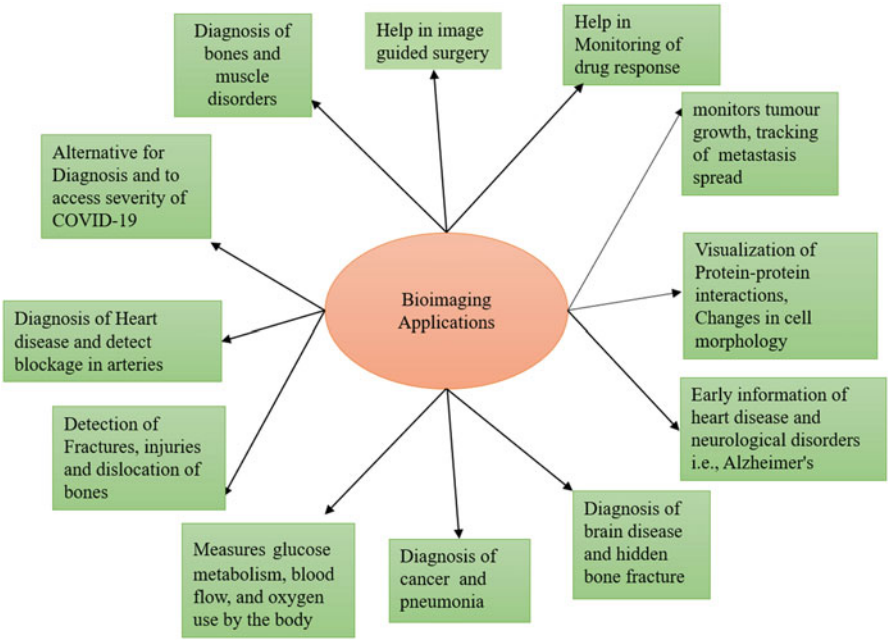

Fig. 2 Applications of bioimaging modalities in modern research

Fluorescence microscopy has challenges like phototoxic effect and photobleaching due to fluorophores, and it is only allowing observation of specific structures that are labeled for fluorescence. But some bioimaging methods should be more developed because bioimaging cannot distinguish between benign and malignant tumors. Till today, no methods can quantitatively give results of response to a drug. There needs to be further exploration of these techniques. No single technique of bioimaging can span the complete resolution spectrum, so there is a need to integrate the information obtained from different modalities. Till now, no such technique based on X-rays has been able to investigate metabolic processes. Techniques such as CT and MRI give good consistent data for diagnosis and analysis of diseases, which reduce image-based biopsies and other invasive procedures. But there is still a need for advancement in CT in the future because contrast agents used in CT cause allergy in some patients. And CT also has a risk of causing cancer due to exposure to ionizing radiation. New generations of MRI cover a wide range of clinical applications, but still, use is challenging due to its complexity and high cost. 18F-FDG is the most commonly used tracer in PET scans for diagnosis and detection of recurrence of cancer, which is a marker for glucose metabolism. However, there is a need to identify specific radioligands to detect tumor-specific features in addition to glucose metabolism markers.

13 Cross-References

- ▶ Emerging Drug Delivery Potential of Gold and Silver Nanoparticles to Lung and Breast Cancers
- ▶ Mammalian Cell Culture Laboratory: Equipment and Other Materials
- ▶ Nanomaterials: Compatibility Towards Biological Interactions

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Troubleshooting of Mammalian Cell Culture

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_24-2

Abstract

Physiologically, healthy culture of cells is the basic need of present mammalian cell culture. During mammalian cell culture, the engaged laboratory personnel may face several troubles/challenges and problems. So, several cautionary measures are necessary to follow to overcome these trouble/challenges and problems. This chapter describes some routine trouble/challenges or problems being encountered by cell culture personnel, amidst the day-to-day laboratory works and the possible remedies to counter these hindrances. The major trouble, a mammalian cell culture personnel is likely to observe involves the changes in the pH of the cell culture medium, nonadherence or null attachment of adherent mammalian cells with the culture vessel surfaces, null or slow cellular growth, clumping of suspension cells in culture, programmed death (apoptosis) of the cultured cells, difficulty in recovering the cells from a cell bank, problems associated with the efficient cell transfection, and finally, contamination of mammalian cell culture with various microorganisms such as mycoplasma, bacteria, fungus, protozoa, and viruses. The success of mammalian cell culture depends upon the understanding of all the basic troubles associated with mammalian cell culture and concurrent effective troubleshooting.

Keywords

Troubleshooting of Mammalian cell culture · Viable/Nonviable cells · Mammalian Cell culture contamination · pH shift of culture medium · Slow

growth of Mammalian cells · Non-adherence of adherent cells · Clumping of Mammalian cells in suspension · Inefficient transfection of Mammalian cells · Freezing and thawing of Mammalian cells

1 Introduction: Troubleshooting in Mammalian Cell Culture

Cell culture is a sterile procedure of growing only desired cells in vitro. Therefore, it is very likely that while a cell culture is in process, different problems are likely to be encountered at varied times. Some problems are common for all users, while others are difficult to address for even experienced workers. While primary cultures face some particular problems, their cause may be different than the same problem encountered in established cell cultures (Arango et al. 2013).

Various mammalian cells may have different nutritional requirements. So, during medium preparation, special attention should be given to adding specific nutrients and growth factors necessary for the culture of specific cells; otherwise, the cells may not grow up to an optimum level. While most mammalian cells are adherent in nature, a few like lymphocytes grow as a suspension culture. The adherent mammalian cells are cultured only on polystyrene-grade culture vessels. Additionally, for strictly adherent cells (e.g., endothelial cells), culture vessels must be coated with adhesive agents such as gelatin, poly-L-lysine, laminin, etc. During cell culture, if adherent cells do not properly adhere to the culture vessels, they are likely to exhibit inadequate growth. Thus, it is highly essential to understand various factors that may adversely affect the adherence or attachment of cells and the process of overcoming these difficulties (Yao and Asayama 2017).

There are several reasons for the situation wherein cultured mammalian cells either do not grow at all or may grow very slowly, or sometimes, may even die due to apoptosis. For example, according to the Hayflick effect, every mortal mammalian cell has a definite capacity for division following which it perishes. Even immortal cells (e.g., cancer cells) may change further due to genetic drifting if passaged continuously (Jafri et al. 2016). If a cell culture is encountered with these problems, the associated person must have adequate knowledge to identify specific problems associated with the growth or apoptosis (of the mammalian cells) along with robust hands-on experience in resolving such bottlenecks (Hayflick and Moorhead 1961; Hayflick 1965).

Finally, mammalian cells are large and grow slowly. In comparison, microorganisms are small single cells and grow much faster. For example, *Escherichia coli,* a Gram-negative bacterium has a doubling time of just 20–30 min (Wang and Levin 2009). In comparison, the fastest growing mammalian cells may have a 10–12 h doubling time (Cooper 2004). The major microorganisms that are likely to contaminate mammalian cell cultures include mycoplasma, bacteria, fungus, protozoa, and viruses. Since microorganisms are omnipresent (in air, water, and surfaces of the floor, instruments, etc.), they can easily contaminate the mammalian cell culture, rapidly consuming all the nutrients, overwhelming the culture, and ultimately destroying it (Chernov et al. 2014). So, the cell culture personnel must have a

thorough knowledge to identify various contamination sources as well as to grow mammalian cells in a contamination-free manner.

Thus, collectively, the common problems associated with the mammalian cell culture may range from optimum medium preparation to nonadherence cell growth, slow culture growth, and unexpected cell death or contamination with other cell types. These problems along with their possible causes and remedies are discussed in this chapter (Geraghty et al. 2014; Yu et al. 2015).

The following pages chronologically describe the various problems associated with* in vitro *mammalian cell culture and associated troubleshooting.

2 Problem 1: Rapid pH Shift in Medium

2.1 Possible Cause # 1

Inadequate carbon dioxide (CO_2) tension.

2.2 Suggested Solution

- The percentage of CO_2 gas in the CO_2 incubator is based entirely on CO_2 gas inflow. CO_2 gas adjusts the concentration of carbonic acid prepared via water and sodium bicarbonate reaction in the medium. For (2.0 to 3.7) $g{\cdot}L^{-1}$sodium bicarbonate concentrations, use (5 to 10)% CO_2 amounts proportionately. This can be accomplished by increasing or decreasing the percentage of CO_2 gas in the CO_2 incubator.
- Adjust the pH of the medium buffering with HEPES during preparation while adding $NaHCO_3$. Adjust the final pH between (7.2 to 7.4) (Michl et al. 2019).
- Switch to a CO_2-independent medium.

2.3 Possible Cause # 2

Excessively tight caps on tissue culture flasks (Yazdani 2016).

2.4 Suggested Solution

Loosen the culture flask or tube caps by one-quarter turn so that CO_2 gas can pass inside.

2.5 Possible Cause # 3

Absence of buffer in the medium due to insufficient bicarbonate content.

2.6 Suggested Solution

Add HEPES buffer to a final concentration of (10 to 25) mM.

2.7 Possible Cause # 4

Incorrect salt composition in the medium.

2.8 Suggested Solution

Use either ***Earle's salts***-based medium or **Hanks' Balanced Salt Solution (HBSS)**-based medium.

2.9 Possible Cause # 5

Contamination by microorganisms such as bacteria, yeast, or fungi.

2.10 Suggested Solution

Start a fresh culture after discarding the complete culture.

If discarding the culture is not possible, decontaminate the culture by adding antibiotics and antimycotics (See Chapter 5).

3 Problem 2: Medium Precipitation with No pH Change

3.1 Possible Cause # 1

After detergent washing of the medium containers, the residual phosphate may be left that precipitates the medium components.

3.2 Suggested Solution

- Do not wash cell culture containers with detergent.
- Immerse all cell culture containers in chromic acid water or water diluted with hydrochloric acid only. Wash extensively with water to eliminate the acid. Rinse the glassware and plasticware thereafter in deionized, distilled water several times followed by sterilization (Bridson and Brecker 1970).

3.3 Possible Cause # 2

Precipitation of the medium salts.

3.4 Suggested Solution

Put the medium to 37 °C to warm it and then swirl to dissolve. However, if precipitate persists, discard the medium. Use freshly prepared medium.

NB: Upon being cooled, the concentrated stock of L-glutamine precipitates. Under these conditions, warming the L-glutamine solution in a 37 °C water bath with gentle swirling will dissolve the precipitate.

4 Problem 3: Medium Precipitation with pH Change

4.1 Possible Cause

Bacterial or fungal contamination.

4.2 Suggested Solution

Discard medium. The precipitate could be due to bacterial or fungal contamination.

Try to decontaminate the culture (see chapter "Culture Decontamination Using Antibiotics and Antimycotics").

5 Problem 4: Adherent Cells Not Attaching to the Culture Vessel

5.1 Possible Cause # 1

Type of cell culture plate(s) or flask used.

5.2 Suggested Solution

Check the type of cell culture container used for adherent and suspended cells. Some manufacturers provide containers with very hydrophobic surfaces that are good for suspension cultures only and are not at all suitable for adherent cultures. Using such containers to culture adherent cells, cells may not efficiently attach to the surface and ultimately perish.

5.3 Possible Cause # 2

No attachment factors in the medium.

5.4 Suggested Solution

- Adherent cells need special attachment factors for improved adherence to the surface of the cell culture container. Some adherent cell lines require a special pre-coating of **poly-L-lysine** (diluted in water), pre-warmed **gelatin** (5% in PBS), **collagens** (diluted in low molar acid solutions such as acetic acid or hydrochloric acid), and **fibronectin** (diluted in calcium- and magnesium-free PBS) to facilitate adherent growth (Refer Chapter 4).
- Especially, for serum-free medium formulations, make sure the medium contains adequate attachment factors like ECGS, heparin, etc.
- Add the diluted coating solution to cover the entire surface of cell culture containers and incubate at 37 °C for an hour. Discard the solution, wash the container with pre-warmed PBS, and finally, seed the adherent cells for attachment.

5.5 Possible Cause # 3

Cells are excessively trypsinized.

5.6 Suggested Solution

- Use diluted trypsin.
- Trypsinize the cells for a short duration and constantly observe through a microscope after adding trypsin to the culture vessel.
- After trypsinization, neutralize the cells from excess trypsin using a complete medium containing 10% FCS followed by at least two times washing with 1X PBS buffer.
- Count and seed the cells as per the standard protocol.
- Now the cells should grow in a surface-adherent manner.

5.7 Possible Cause # 4

Mycoplasma contamination.

5.8 Suggested Solution

- Test for mycoplasma contamination.
- Clean the CO_2 incubator and laminar flow hood.
- If contaminated, discard the culture.

(*See chapter* "Decontamination of Cultures with Mycoplasma")

6 Problem 5: Poor Viable Cell Count After Thawing the Stock

6.1 Possible Cause

- Cells were stored incorrectly.
- Cells were excessively passaged.
- Cells were thawed incorrectly.
- Either wrong cell culture medium composition or an inadequate pre-warming.
- Cells were diluted.
- Harsh cell handling during and after thawing.

6.2 Suggested Solution

- Obtain a new stock and use slow freezing in subsequent attempts.
- Excessively passaged mortal/primary cells become senescent.
- Even immortal cells exhibit **genetic drifting** after several passages. So, one should use **ATCC recommended protocol for cell passaging**.
- Thawing rapidly decreases the ice crystals' effects in the freeze-dried cells.
- Centrifuge and resuspend the cells. The obtained cells might be pro-apoptotic owing to harsh handling. So, it is wise to start with new cells.

7 Problem 6: Cells Are Not Growing

7.1 Possible Cause # 1

The type of culture medium for optimum cell growth may not be proper.

7.2 Suggested Solution

When cells are not growing at all, always check first in the repository of the biggest cell banks (ATCC and ECACC) whether the used medium is recommended for that particular cell type.

Recommendations of the medium for the particular cell can be also obtained from previous scientific publications reporting about selective cell line propagation.

7.3 Possible Cause # 2

Requirement of nutritional supplements for optimum cell growth.

7.4 Suggested Solution

Many of the known cell lines need additional nutritional supplements for optimum growth. The most commonly used nutritional supplements are fetal bovine serum (FBS) or fetal calf serum (FCS), typically at (10 to 20)% (v/v) concentration, L-glutamine besides nonessential amino acids (NEAA) (Rubin 2019; Sedelaar and Isaacs 2009; Salazar et al. 2016).

7.5 Possible Cause # 3

Too low initial cell inoculum.

7.6 Suggested Solution

Most adherent cells need cell-to-cell contact for initial growth, and therefore, minimum cell number in the inoculum is needed for occupying a certain area of the cell culture container. Therefore, one should allow the growth of recommended threshold cell population, depending on the characteristic flask or plate size. Only then cells will grow to yield a confluent culture (Chapter 8–10: Refer to published manuscripts or manufacturer's recommendation for cell container-specific cell inoculum).

7.7 Possible Cause # 4

Stock cells are not in good physiology due to improper freezing.

7.8 Suggested Solution

Use a fresh vial of cells and separately freeze the stock.

8 Problem 7: Decreased or Slow Culture Growth

8.1 Possible Cause # 1

There may be changes in the serum (FBS/FCS) or medium.

8.2 Suggested Solution

- Analyze the medium formulations for glucose, amino acids, and other components.
- The old and new batches of serum (FCS/FBS) should be compared for growth.
- The seeding manner might have increased the number of cells.
- Cells should be sequentially adapted to a new medium.

8.3 Possible Cause # 2

- One may have missed adding L-glutamine to the cell culture medium.
- L-glutamine or other growth additives such as growth factors break down or are degraded (Rubin 2019; Duffy et al. 2000–2013).
- The expiry date of the medium or the associated compounds might have long passed.
- Wrong content of medium supplements due to chance calculation mistakes.

8.4 Suggested Solution

- Replace the old medium with the freshly prepared medium.
- If the problem persists, add L-glutamine and other growth additives including growth factors in the culture medium.
- Substitute GLUTAMAX I or II for L-glutamine in the medium.

8.5 Possible Cause # 3

Low-level bacterial or fungal contaminations.

8.6 Suggested Solution

Grow culture without antibiotics and discard in case of contamination.

Try to decontaminate (see chapter “Decontamination of Culture with Antibiotics and Antimycotics”).

8.7 Possible Cause # 4

Improper reagent storage.

8.8 Suggested Solution

- Aseptically aliquot the medium and serum.
- Store the serum aliquots at −20 °C.
- Store the medium aliquots at 2–8 °C.
- Store the complete medium in the refrigerator (2–8)°C and use it within 1 week.
- Minimize exposure of sera and medium to light and room temperature (RT).

8.9 Possible Cause # 5

Excessively low initial cell inoculum.

8.10 Suggested Solution

Increase the number of viable cells in the beginning inoculum as cells fervently require cell-to-cell contact for survival (Refer to published manuscripts or manufacturer's recommendation for knowing about cell inoculum depending on the container size).

8.11 Possible Cause # 6

Senescence of finite culture/excessive cell passaging.

8.12 Suggested Solution

Discard culture and start the new culture with fresh cell stock.

8.13 Possible Cause # 7

Mycoplasma contamination.

8.14 Suggested Solution

Segregate culture and test for mycoplasma presence in the culture. Clean the laminar flow hood and CO_2 incubator. Discard the culture if it is contaminated.

8.15 Possible Cause # 8

Cells were too confluent at the time of being passaged.

8.16 Suggested Solution

Start with a new cell stock and freeze early to mid-log phase.

8.17 Possible Cause # 9

The prevailing CO_2 content of the medium does not match the one needed by the bicarbonate-based buffering system, inevitably caused due to impaired pH control.

8.18 Suggested Solution

- Adjust the medium pH (7.2 to 7.4) either by adding bicarbonate solution or HEPES buffer. CO_2 is not a metabolic requirement of cell cultures and is exclusively prevalent in the dissolved form in a cell culture medium where a little extent of it forms carbonic acid (H_2CO_3) on reacting with water. The carbonic acid decomposes into H^+ and HCO_3^-.
- The added $NaHCO_3$ in the medium decides the corresponding CO_2 needed for pH maintenance.
- **NB:** Most of the mammalian cell culture medium including Eagle's Minimum Essential Medium (EMEM) is supplemented with Earle's Balanced Salt Solution (EBSS), formulated with 26 mM $NaHCO_3$. On the other hand, the DMEM has 44 mM $NaHCO_3$, much higher than most of the medium.
- The bicarbonate buffering action is the most efficient pH controlling mechanism (though there are several other synthetic buffers functional over a wide range of laboratory conditions), owing to its exclusive activity within the body. Owing to this, the bicarbonate buffer system is used for in vitro cell culturing to mimic human and mammalian physiology via ensuring minimized side effects.
- Readers must understand that bicarbonate buffering works via ***Le-Chatelier's principle*** (maintaining status quo concerning physiological status corresponding to any sort of external modulation), whereby increased medium acidity is contributed by enhanced H^+ concentration with free HCO_3^- reacting with extra H^+ to

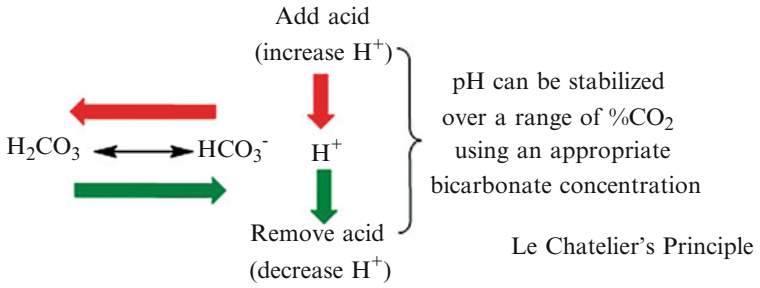

Fig. 1 Regulation of cell culture medium pH through bicarbonate buffer

form H_2CO_3. Concomitantly, the reaction is shifted to the left, and pH is stabilized, resulting in shifting the equilibrium to right (Fig. 1).

- A decline in the pH of the growth medium infers a buildup of lactic acid, generally a cellular metabolism by-product. Lactic acid could be toxic to the growing cells while the decreased pH may be suboptimal for cell growth.
- In general, cell culture media are supplemented with the indicator dye Phenol Red, wherein a yellow hue in the medium infers a too low pH whereas a purple hue predicts a too high pH. Orange-reddish hue is the sole indication of an optimum physiological status of the medium. More so, a high cell population is likely to consume the culture media early, and this could be compensated by subculturing the cells as soon as a quick pH decline ($>$ 0.1 to 0.2 units) is noticed concerning increasing cell concentration.

9 Problem 8: Death of Cultured Cells

9.1 Possible Cause # 1

No CO_2 gas in the incubator.

9.2 Suggested Solution

Closely monitor the rate of CO_2 flow in the incubator and check whether the manual readings concur with those displayed on the incubator.

- Check the line connections for any possible leakage.
- Avoid frequent opening and closing of incubator doors so that inside CO_2 concentration reaches 5% and remains at par for stabilizing the medium pH (Gladstone et al. 1935).

9.3 Possible Cause # 2

Inside the incubator, check the temperature with the help of a thermometer.

9.4 Suggested Solution

- Monitor the temperature of the incubator promptly, keeping a thermometer inside.
- Remove the cells to another incubator to keep cells healthy in case of temperature change.
- Repair the incubator.

9.5 Possible Cause # 3

Use of Fungizone at toxic concentrations for getting rid of contamination.

9.6 Suggested Solution

Use a limited amount of Fungizone as it is fatal to cells (see chapter "Use antibiotics and antimycotics in Appropriate Concentrations for Decontamination").

9.7 Possible Cause # 4

Cells are damaged during thawing or cryopreservation.

9.8 Suggested Solution

- Obtain a new aliquot of cryopreserved cells to start a new cell culture batch.
- Rapidly thaw cryopreserved cell aliquots, quickly put them in a warm medium and wash with 1X PBS twice to remove the toxic DMSO. Resuspend the cells immediately in a complete medium after washing (refer to chapter "Cryopreservation of Mammalian Cells").

9.9 Possible Cause # 5

The buildup of non-optimum osmotic pressure in the medium.

9.10 Suggested Solution

Cells burst and die in a hypotonic medium and shrink in the hypertonic medium. Therefore, it is mandatory to examine the osmolality of the complete medium.

NB: Most mammalian cells can tolerate (260 to 350) mOsm•kg^{-1} osmolality. The addition of reagents such as HEPES and drugs may affect osmolality (Shah and Mandiga 2020; Lee and Koo 2010).

9.11 Possible Cause # 6

Inappropriate humidity.

9.12 Suggested Solution

Check the water level in the water pan. Humidity is vital to adequate gas exchange for many cells and media (i.e., appropriate CO_2 levels are substantially irrelevant for most cultures if the humidity is not high enough).

9.13 Possible Cause # 7

The buildup of toxic metabolites in the medium.

9.14 Suggested Solution

Remove the medium and wash with 1X PBS buffer two times before adding the fresh medium.

NB: The concentrations of glutamine and/or asparagine in the being handled cell culture media should be assessed and restricted as both these amino acids can be deaminated and eventually form ammonia (NH_3) as the by-product. During the late stationary phase of mammalian cell culture, cells may utilize alanine to generate pyruvate and this process also produces NH_3. Table supplementation of pyruvate in the culture medium is a possible solution to alleviating NH_3accumulation. Additionally, the issue can be potentially addressed from a process perspective **via** ***a temperature shift scheme or adjusting the harvest time*** (Slivac et al. 2010; Freund and Croughan 2018).

9.15 Possible Cause # 8

Contamination by microorganisms.

9.16 Suggested Solution

Bacterial and fungal contaminations are usually easily visible; symptoms of mycoplasma contamination are more subtle. Careful monitoring of culture morphology and regular testing are necessary to detect such contamination (Herman and Pauwels 2014).

9.17 Possible Cause # 9

An inappropriate medium is being used.

9.18 Suggested Solution

Double-check that the medium used is appropriate for your cell type and culture application. For example, ensure that the medium being used for serum-free culture is designed for serum-free culture. Therein, it must ascertain that the desired drugs are used at appropriate levels alongside checking the expiration dates for the reagents being used and storing the medium at appropriate temperatures in the dark (Even et al. 2006; Niepel et al. 2017; Neutsch et al. 2018).

NB: Apoptosis is typically caused by nutrient depletion and/or metabolite accumulation. Nutrient depletion can be prevented by proper feeding strategies. Metabolite accumulation is less of a concern in a perfusion process but is often challenging to tackle in a fed-batch process. Media optimization approaches to control the accumulation of metabolites may include: reducing glutamine to control ammonia, reducing bicarbonate to modulate CO_2, and using alternate carbon sources to control lactate levels. In addition, there are reports on targeting the apoptosis pathway by incorporation of chemicals such as caspase inhibitors or the use of silencing RNA. However, these approaches have not been incorporated into industrial processes yet.

10 Problem 9: Mutual Clumping of Suspension Cells

10.1 Possible Cause # 1

Presence of calcium and magnesium ions.

10.2 Suggested Solution

Wash the cells in a balanced salt solution not containing calcium and magnesium. Gently resuspend the cells with sterile tips or Pasteur pipettes to get a single cell suspension.

10.3 Possible Cause # 2

Mycoplasma contamination.

10.4 Suggested Solution

Isolate the culture and test for mycoplasma infection. Clean hood and incubator. If the culture is contaminated, discard the culture and start afresh (see chapter "Decontamination of Cultures with Mycoplasma").

10.5 Possible Cause # 3

Cell lysis and release of DNA resulting from over digestion with proteolytic enzymes.

10.6 Suggested Solution

Treat clump of dead cells with DNase before centrifugation followed by twice PBS washing. Once cells are clump-free, count their viable population in the pellet and culture them in a fresh medium (see chapter "Proteolytic Degradation of Attached Cells").

11 Problem 10: Primary Cell Culture Is Contaminated

11.1 Possible Cause

Contaminated primary tissue is processed in the culture.

11.2 Suggested Solution

- Discard the contaminated culture. Start afresh by isolating primary tissue again and setting up fresh culture with all precautions.
- In case of the primary tissue unavailability, wash tissue pieces several times with salt and PBS or BSS containing a higher concentration of antibiotics and antimycotics before starting the culture.
- (*See chapter* "Decontamination of Cultures with Antibiotics and Antimycotics").

12 Problem 11: Cells Are Regularly Contaminated with Mycoplasma or Other Pathogens

12.1 Possible Cause

Contamination of culture by pathogens or mycoplasma amidst non-sterile handling of cell culture.

12.2 Suggested Solution

- Work with cells under cell culture hoods. Use sterile tips and pipettes, and sterilize the glassware and tools by autoclaving.
- Follow completely aseptic procedures during cell culture.
- Wear appropriate PPE, including gloves and a lab coat.
- Clean and decontaminate the working area.
- Discard the contaminated culture and start the culture afresh from the master stock.
- For precautionary measures, maintain two cell cultures, one with antibiotics and one antibiotic-free. Examine both cultures regularly for any signs of infection.

Note: ***Regular use of antibiotics in routine cell culture can mask low-level infections that are barely detectable. Antibiotics may provide selective pressure for developing antibiotic-resistant pathogens in the working area. Therefore, the use of antibiotics should be limited. Always perform routine microbiological screening of the working and master stocks to ensure that they are free of contamination.***

13 Problem 12: Difficulty in Recovering Any/Many Cells from the Cell Repository

13.1 Possible Cause

Problems in freezing and thawing of cells.

13.2 Suggested Solution

- Always freeze cells slowly and thaw cells rapidly to avoid cell death.
- Follow the procedure of freezing and thawing cells properly (Refer to chapter "Cryopreservation of Mammalian Cells").
- Make sure all frozen vials are stored below −130 °C in liquid versus vapor phase nitrogen at all times.
- Always freeze multiple vials at a time and at least 1 x 10^7 live cells per vial.

- Always determine the % viability of freshly thawed cells.
- After thawing seeds high number of cells, allowing rapid logarithmic growth to acquire cells in higher density.

14 Problem 13: Problems with Inefficient Transfection of Cells

14.1 Possible Cause

Problems due to transfection efficiency.

14.2 Suggested Solution

- Efficient cell transfection depends on the passage number and confluency of the cells. Use early passage cells and start transfection at (50–70)% growth confluency.
- Always carry out cell transfection in absence of antibiotics and serum as they can diminish transfection efficiency. Cells should be serum-starved before transfection.
- Optimize transfection protocols for every cell line.
- The transfection efficiency of cells can vary depending on a particular transfection reagent, the ratio of DNA/RNA to transfection reagent, and the post-transfection cell recovery.

14.3 Successful Cell Culture Tips to Avoid Troubleshooting

14.3.1 Cell Authentication and Source Validation

- Choose a cell carefully so that it possesses the desired biological parameters, and can tolerate the experimental designs apart from generating reproducible results.
- Always procure the cells from a trusted, authentic source such as ATCC with good quality control along with formal documentation that includes the cell's identity, its specific type, and typical growth conditions, just like the medium used for growth.
- Quarantine any new cell obtained until it is authenticated to avoid contamination with other preexisting cells in the laboratory.
- For clinical samples, strictly follow all ethical and legal guidelines including the patient's consent for the acquisition of the tissue sample. Always record the source and clinical information of the donor or patient for generation of the cell line from the tissue, cell type, lot numbers of all reagents used, culture images, and genetic modification methods, if performed.
- For cells obtained from clinical samples, always preserve a tissue sample for histopathology as well as a sample of normal tissue for comparison.

14.3.2 Existing Cells Currently in Use

- Characterize the cells at regular intervals to promptly ascertain any random variability in genotypic and phenotypic instability. In case of any doubt, discard the old ones and replace them with original frozen stock.
- Monitor the identity of cells using DNA genotyping, at least once per year and ideally, every 6 months. Comparison of short tandem repeats (STRs) or single nucleotide polymorphisms (SNPs) with available databases is the most cost-effective and acceptable genetic method for intraspecies distinction.
- Always handle one cell at a time to avoid mixing, and label the cells, one at a time to maintain the true identity of cells throughout.
- Mixing of cells may happen mostly during frozen cell storage in liquid nitrogen vapor interphase due to mislabeling or removal of labels etc.
- For precautionary measures, always check with the International Cell Line Authentication Committee (ICLAC) registry of misidentified cell lines (Horbach and Halffman 2017).

14.3.3 Safe Work Practices

- Proper training of all personnel engaged in cell culture is mandatory. Follow standard operating procedures (SOPs) for culturing all the cells and ensure strict implementation for all laboratory personnel. All personnel should learn good laboratory practices (GLP) and aseptic cell-culturing procedures according to the required biosafety level in the cell culture facility (Nambisan 2017; Cooper-Hannan et al. 1999; Laboratory Biosafety Manual, WHO 2004).
- A subclause under the umbrella of GLP is GCCP (Good Cell Culture Practices), the first guidance of which dates back to 2005. The rulings demonstrated thereby relate to quality assurance aspects for in vitro cell culture, focused on diversified cell types and culture formats useful in the research, product development, testing, and manufacture of biotechnology products. The guidelines encompass a basic code of conduct to be followed in training new personnel, reviewing and improving experimental aspects, besides working towards assured standard practices and conditions for comparing the laboratory results with experiments performed at different times (Pamies et al. 2017).
- To avoid mixing and cross-contamination, never work on more than one cell line at a time.
- Do not share bottles of media between cell lines. Always make a working medium in a separate container from the main stock, and after work, discard the residual content of working.
- Prepare and filter the medium either on the same day or at least 2 days before commencing actual work. Watch for contamination, keeping one small aliquot at 37 °C for at least (24–48) hours. If contaminated, then discard the original stock and restart fresh. If the original stock is frozen, then consider filtering again and testing the aliquot before proceeding forward.
- Serum quality is an important determinant in cell culture. Test each batch to make sure the serum is suitable for optimum growth. Record the serum batch and lot number, particularly for future troubleshooting.

- Always add supplements like L-glutamine, nonessential amino acids (NEAA), etc. freshly just before work. Purchase cell culture grade high-quality, fine reagents from reputed manufacturers ensuring good quality control to avoid any problem in cell culture besides generating consistent data (Rubin 2019; Salazar et al. 2016) (Table 1).
- For their healthy growth and development, adult vertebrate animals require nine amino acids which cannot be synthesized within their bodies, and hence, have to be obtained from the diet. Animal cells grown in culture also require these amino acids which include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Table 1).
- Apart from these nine amino acids, most cultured cells also require cysteine, glutamine, and tyrosine. In a healthy mammal, these amino acids are prepared by specialized body cells. For instance, liver cells harness tyrosine from phenylalanine.

Table 1 Aqueous solubility and optimum concentration range of essential and nonessential amino acids needed for mammalian cell culture

Amino acid	Solubility at 25 °C ($g \cdot kg^{-1}$ H_2O)	Charge at pH 7	Concentration range in culture medium ($g \cdot l^{-1}$)
Essential amino acids			
L-Arginine	182.60	+(−/+)	0.084–1.331
L-Cysteine		+/−	0.024–0.123
L-Cystine	0.11		0.031–0.115
L-Glutamine	42.00	+/−	
L-Histidine	43.50	+/−	0.015–0.152
L-Isoleucine	34.20	+/−	0.050–0.457
L-Leucine	23.80	+/−	0.050–0.560
L-Lysine	5.80	+(−/+)	0.000–2.000
L-Methionine	56.00	+/−	0.015–0.153
L-Phenylalanine	27.90	+/−	0.015–0.313
L-Threonine	90.60	+/−	0.020–0.750
L-Tryptophan	13.20	+/−	0.005–0.080
L-Tyrosine	0.51	+/−	0.029–0.197
L-Valine	88.00	+/−	0.020–0.440
Nonessential amino acids			
Glycine	239.00	+/−	0.008–0.330
L-Alanine	166.90	+/−	0.009–0.318
L-Asparagine	25.10	+/−	0.026–0.589
L-Aspartic acid	5.04	–	0.013–0.465
L-Glutamic acid	8.60	–	0.011–0.642
L-Proline	1625.00	+/−	0.000–0.121
L-Serine	250.00	+/−	0.030–0.557

- The remaining eight amino acids can be synthesized from the cells within the organism or that are being cultured. So, these amino acids need not be essentially present in the diet or culture medium.
- Perform regular calibration and servicing of all equipment, including pipettes, microscopes, and autoclaves without failure. For maintenance of the aseptic environment, sterilize and decontaminate the laminar flow culture hood and CO_2 incubator on regular basis (Çelik-Uzuner and Uzuner 2017).
- At every final stage, viable cells should be counted before going for further experimentation or cell storage.
- Before cell counting, mix the cells thoroughly to get a homogeneous mixture that gives a reproducible and error-free cell count. During manual cell count by hemocytometer, take extra care while loading the chamber to avoid accidental interference by air bubbles for uniform cell distribution and cell count (Cadena-Herrera et al. 2015; Lombarts et al. 1986).
- Count the cells preferably using an automated Coulter principle-based counter so that cells are not only counted but average cell size and distribution pattern of the cell population could also be displayed.

14.3.4 Preventing Microbial Contamination

- The use of antibiotics and antimycotics can mask the indications of low-grade infections. In an antibiotics-free medium, cloudiness, as well as change in medium color and pH, indicates the presence of bacterial, yeast, or fungal infection (Chiarini et al. 2008; Nawrot et al. 2015).
- It is worth noting herein that mycoplasma infection pertains to a serious contamination source on in vitro cell cultures, manifesting as genetic instability, transformation, alterations in physiological functioning, and viral susceptibility. The threatening consequence of mycoplasma infection is its inherent resistance to certain antibiotics (due to meager cell wall material) along with the ability to pass through microbiological filters with null effect on cell growth and consequential pH changes in the culture medium (Bruchmuller et al. 2006; Hay et al. 1989).
- Use a polyethersulfone (PES) membrane with a 0.22 μm pore size for filtering medium containing serum and other proteins. Ideally, after the addition of serum or any other proteins, it is mandatory to perform double filtration using **a 0.22 μm** membrane (Unger et al. 2005).
- Mycoplasma infection in a culture often does not show any visible signs of contamination but can be present to a significant extent. Use a PES membrane with a 0.10 μm pore size to remove contaminating mycoplasma from the medium.
- Use a polyvinylidene difluoride (PVDF) membrane with a 0.2 μm pore size for very low protein binding and sterilizing solutions having a high extent of biomolecules.
- Several antibiotics could also be included in the culture media to arrest the mycoplasma contamination. However, with continued use, several traditional antibiotics manifest a resistant response. So, caution must be exercised regarding the antibiotic inclusion with small pre-sampling so that there are no unusual toxic responses or side effects (Tables 2 and 3) (Nikfarjam and Farzaneh 2012).

Table 2 Selective anti-mycoplasma antibiotics with their brand and generic names and corresponding antibiotic class

Generic name	Brand name	Antibiotic category
Tiamulin (BM-cyclin 1) Minocycline (BM-cyclin 2)	BM-cyclin	Macrolide Tetracycline
Unknown	Plasmocin	Tetracycline and quinolone
Ciprofloxacin	Ciprobay	Quinolone
Enrofloxacin	Baytril	Quinolone
Sparfloxacin	Zagam	Quinolone
Unknown	MRA	Quinolone

Table 3 Antibiotic resistance of mycoplasma from infected cell cultures

Antibiotic	Resistance (in %)
Chlortetracycline	11
Spectinomycin	14
Tetracycline	14
Ciprofloxacin	15
Tulosin	21
Lincomycin	28
Chloramphenicol	30
Kanamycin	73
Gentamycin	80
Neomycin	86
Streptomycin	88
Erythromycin	98

- Some of the toxic manifestations of mycoplasma are inhibition of cell growth and metabolism, compromised viral production, reduced transfection efficacy, and cell death.
- These alterations in cultured pattern and metabolism of the mammalian cells are due to mycoplasma-induced changes in gene expression, DNA fragmentation of the mammalian cells by mycoplasma, alteration of the membrane integrity of the mammalian cells, and other reasons.
- Besides microorganisms (mycoplasmas, bacteria, fungus, protozoa, etc.), the plasticizers eluted from the plastic containers and materials present in the water/cell culture medium may affect the growth of the mammalian cells (Yao and Asayama 2017).

NB: Apart from "known" contaminants such as viruses, bacteria, mycoplasma, and endotoxins, the plasticizers eluted from plastic instruments, or a range of substances present in water, can also contaminate the cells in a culture apart from affecting their behavior (Yao and Asayama 2017).

14.4 Cell Freezing and Thawing

- Always freeze cell samples (for both mortal cells and immortal cancer cells) of the lowest passage as stock so that new culture can be started afresh with consistent and reproducible biological parameters.
- Cells can be frozen during (70 to 90)% confluency but not at 100% confluency since this would impair optimum cell growth due to contact inhibition and other factors. Confluency refers to the percentage of culture vessel surface area covered by a layer of cells upon being observed by a microscope. For instance, 50% confluence infers half of the culture flask or container surface is covered in cells.
- Confluence-aided contact inhibition of cells may cease the growth in some cells although this is less frequent in standard cell culture. The instant at which cultured cells become more than desired or needful confluent, it is implied that native signaling networks are altered, leading to the progressive change of cells into aberrant form and consequent irreproducible results.
- Adherent cultures should be passaged generally in the log phase before reaching confluence. Normal cells cease to grow upon reaching confluence, and it takes them fairly longer to recover upon being reseeded. Transformed cells, on the contrary, continue proliferating even after attaining confluence but often perish after approximately two doublings. Likewise, cells in suspension generally require passaging in the log phase of the growth before reaching confluency. Upon confluency attainment, cells grown in a suspension mode clump together resulting in a turbid appearance of the medium the instant when the culture flask is swirled.
- Count the live cell number before cell freezing. Always freeze cells that are (95–100)% viable. Both adherent and suspension culture cells can be frozen and stored in liquid nitrogen for extended durations. A similar protocol is followed for both cell types.
- Harvest the cells before centrifugation to spin them down. Resuspend the cells diligently using a Pasteur pipette in a cold medium comprising 10% FBS to a concentration of nearly $5{\cdot}10^6$ cells per ml. Keep the tube or bottle containing cells on ice in a sterile hood. Add a volume of cold freezing medium, equivalent to that of (10% FBS) containing cold medium dropwise to the tube with cells while gently spinning the tube.
- Distribute the cell suspension in 1 ml aliquots in 2 ml cryovial. Tighten the caps properly and place the vials in a Styrofoam box inside a −80 °C freezer overnight before transferring them to a liquid nitrogen storage tank.
- As there is a high possibility of mixing cells during freezing, preserve extreme caution during cell vial labeling.
- Label the stock vials with full name, date of freezing, passage number, etc. completely so that it is legible and keep a record in the notebook.
- Do not use labels with glue for freezing cells in liquid nitrogen. Always use permanent markers for labeling so that the information is not easily removed. The labeling should be legible and clear so that the cell details can be read without ambiguity (Freshney 2005).

- Though cells can be stored in liquid nitrogen or gas phase (over the liquid nitrogen) for consistently long durations, valuable cells should be thawed and revived into culture form once a year to ascertain their viability and function.
- For thawing, frozen vials with cells are placed on ice directly from the liquid N_2 storage tank. The vials should be immersed in a water bath at 37 °C and thawed leaving a small ice lump in the vial before transferring it to melting ice again.
- After thawing, wash cells thoroughly with a medium containing 5% serum to avoid any shock.
- Count the viable cells after recovery and before seeding in the new culture. Always record the % recovery of viable cells to determine the freezing efficiency following the cell culture procedure. If cell viability is below 70%, it may be rendered difficult to attain adequate cell growth. Reduced cell viability is most probably caused by improper cell handling before freezing, during freezing, or while thawing.
- Dilute with complete medium to the desired cell concentration and add the cell suspension to a suitable culture vessel. Place in an incubator and monitor the cell growth and morphology up to a few following days.
- For an easier understanding, Fig. 2 depicts the chronological steps in the freezing and thawing procedures of cell line preservation. In general, freezing is performed before thawing and is characterized by progressive (must not be instantaneous) cooling of cells to be preserved.

15 Conclusions

Mammalian cell culture personnel must have all the basic knowledge regarding the trouble or difficulties that may arise during the culture of mammalian cells and also must have enough expertise either to avoid those difficulties or to successfully troubleshoot all such bottlenecks. This chapter systemically described the various basic difficulties or troubles one may face while laboratory culturing mammalian cells. While going through the chapter, the readers may have acquired sufficient knowledge about the manifold troubles in mammalian cell culture, substantiating selecting improper cell culture containers that are unsuitable for culturing adherent cells, failing to choose proper cell culture medium and growth additives that may not be suitable for the specific cells being cultured, altered CO_2 level or changes of medium pH (due to various reasons), harsh handling conditions that may damage the mammalian cells, and finally, contamination of mammalian cell culture with various mycoplasma, bacteria, fungus (yeast/mold), and viruses. Successful mammalian cell culture depends on how a well-trained and experienced cell culture practitioner can either avoid any trouble during cell culture or effectively troubleshoot any difficulties, as and when it appears during any cell culture experiment.

NB: The contents of this chapter have been collected from Gibco Invitrogen Cell Culture Basics: Troubleshooting in Cell Culture; Thermo Fisher Scientific: Cell Culture Troubleshooting Guide; Proteintech: The complete guide to cell

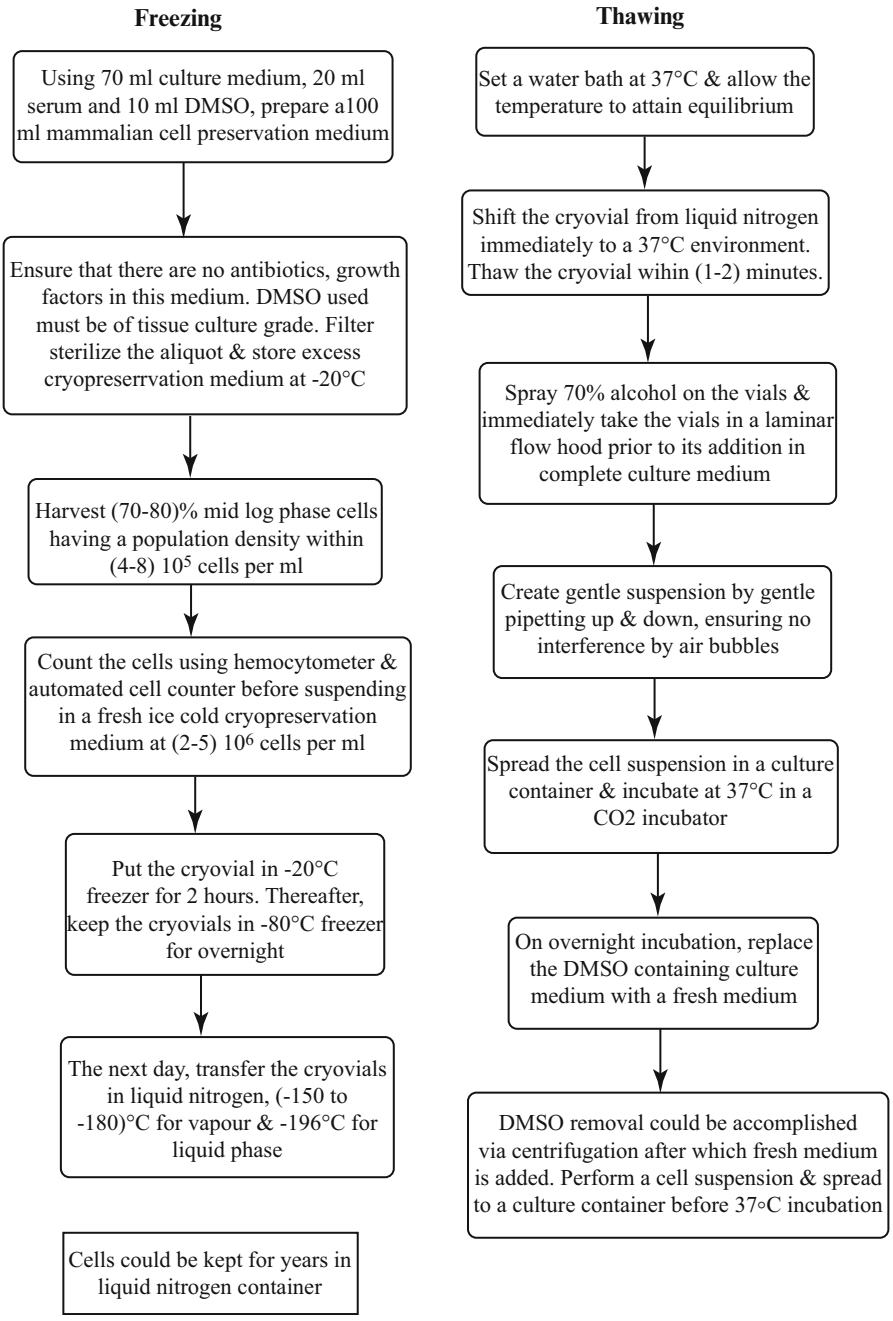

Fig. 2 Flowchart of steps involved in cell line preserving freezing and thawing procedures

culture and Sigma Co. The protocols are modified here and there based on our research experience.

16 Cross-References

► Microbial Contamination of Mammalian Cell Culture

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8

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