Mammalian Cell Culture Types and Guidelines of Their Maintenance

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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:

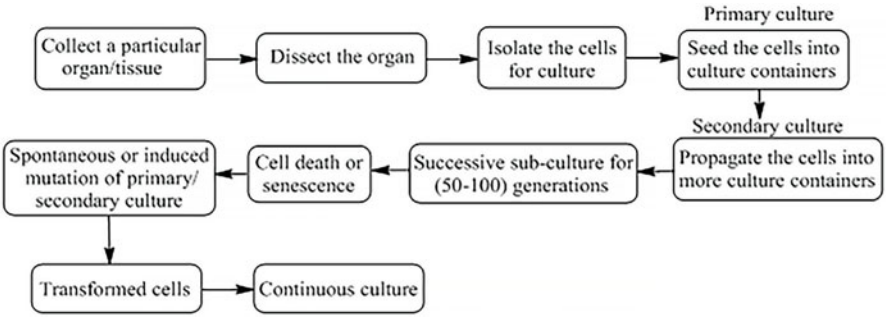

Fig. 1 Schematic representation of primary, secondary, and continuous mammalian cell cultures, typically distinguishing the handling populations at an instant

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:

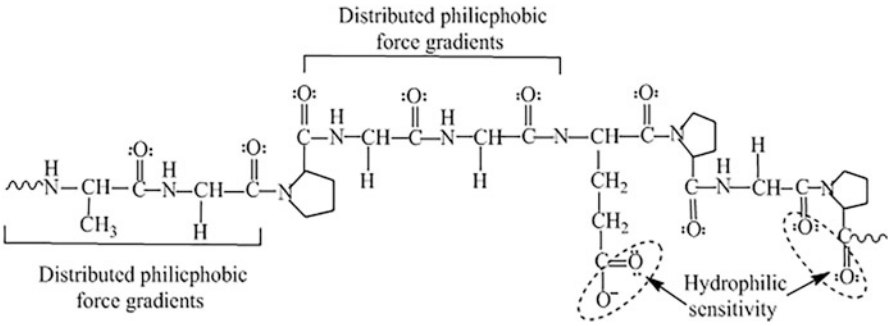

Fig. 2 Chemical structure of gelatin, depicting the characteristic philicphobic force gradients for its aggregation-forbidding abilities

- 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.**

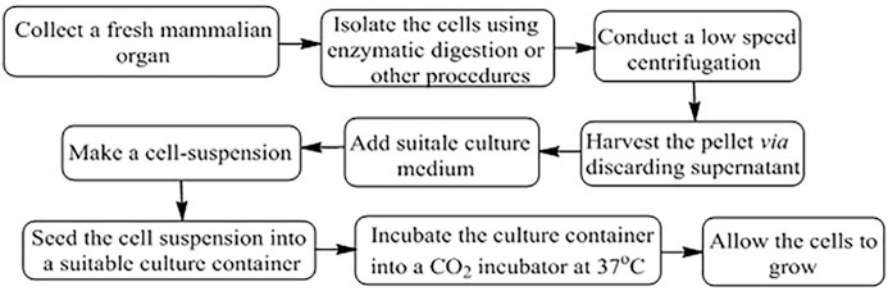

Fig. 3 Schematic representation of mammalian cells isolation and culture procedures

- 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).

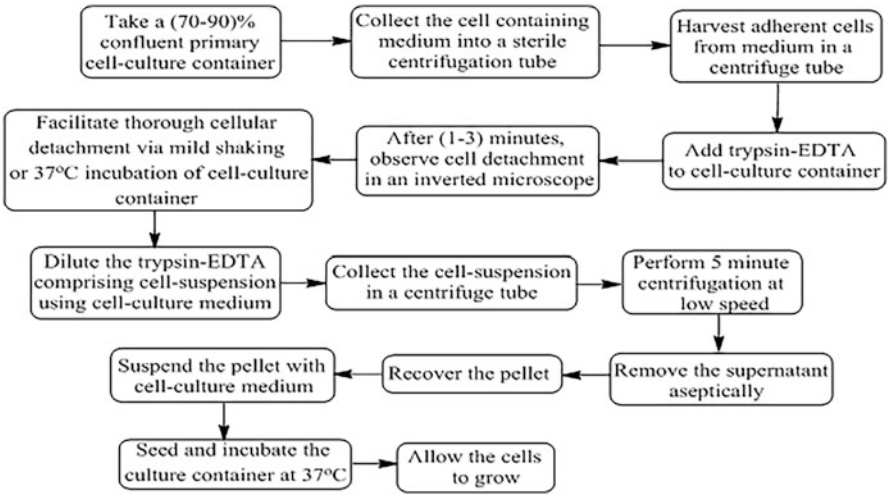

Fig. 4 Schematic representation of primary culture cells subculturing enabling formation of secondary culture cells. **The details of subculturing are described in the propagation of adherent and nonadherent cell culture section**

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.

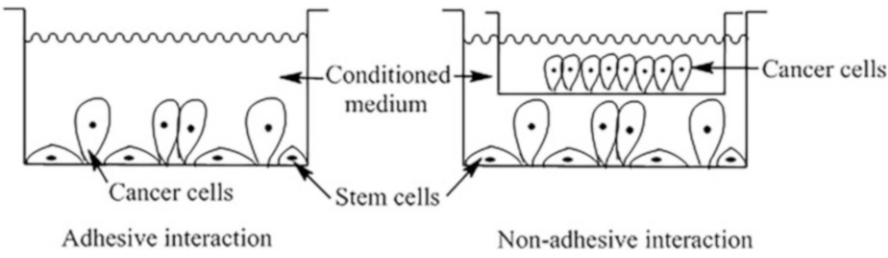

Fig. 5 Cancer cells grow in adhesive and nonadhesive interactive modes

- 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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