Mammalian Cell Culture Laboratory: Equipment and Other Materials

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Contents

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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.

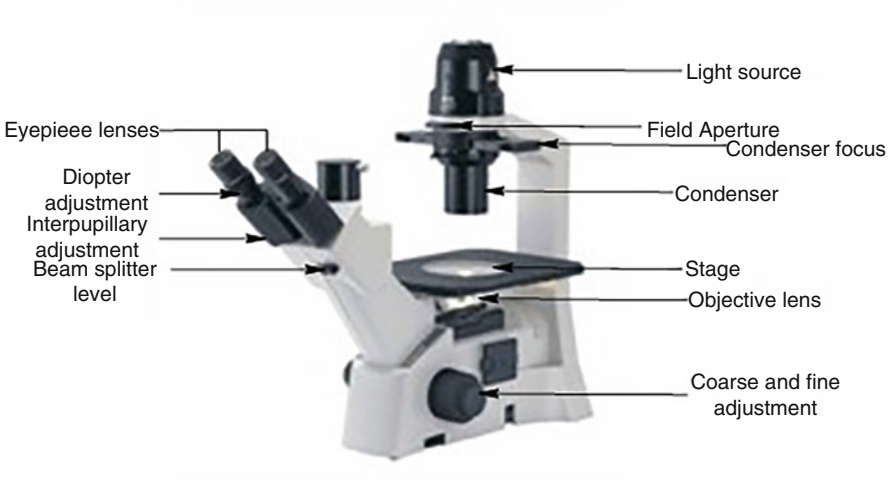


Fig. 6 Description of an inverted microscope

- 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

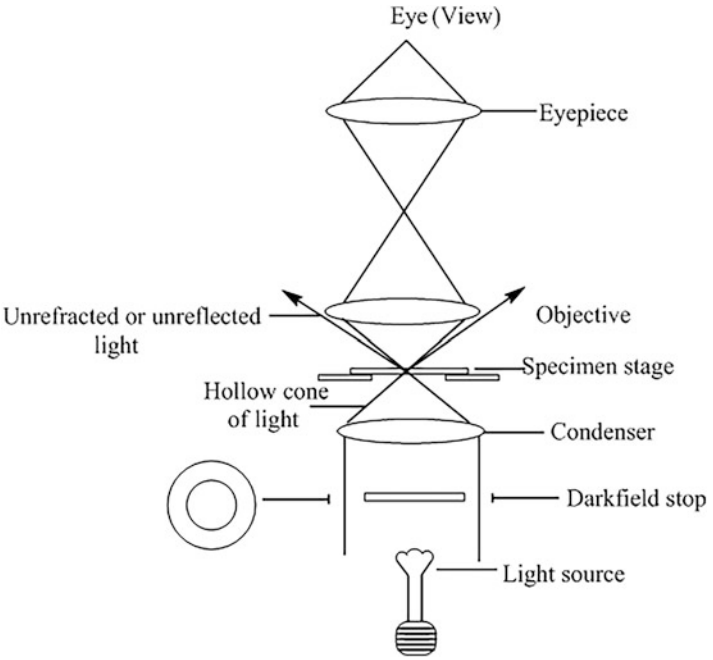


Fig. 7 Working module of a bright-field microscope

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).

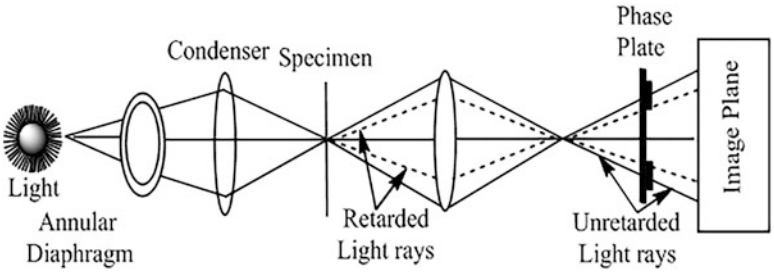


Fig. 8 Working module of a phase contrast microscope

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).

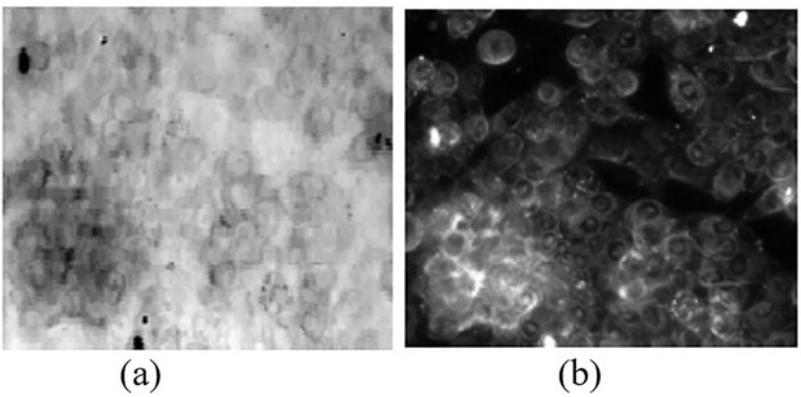


Fig. 9 Imaging of HeLa cells viewed using (**a**) bright-field and (**b**) dark-field modes, in a 20× numerical aperture, 0.4 objective lenses. The unstained biological samples exhibit low contrast in bright-field mode owing to inadequate natural pigmentation. Dark-field visualization enables contrast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

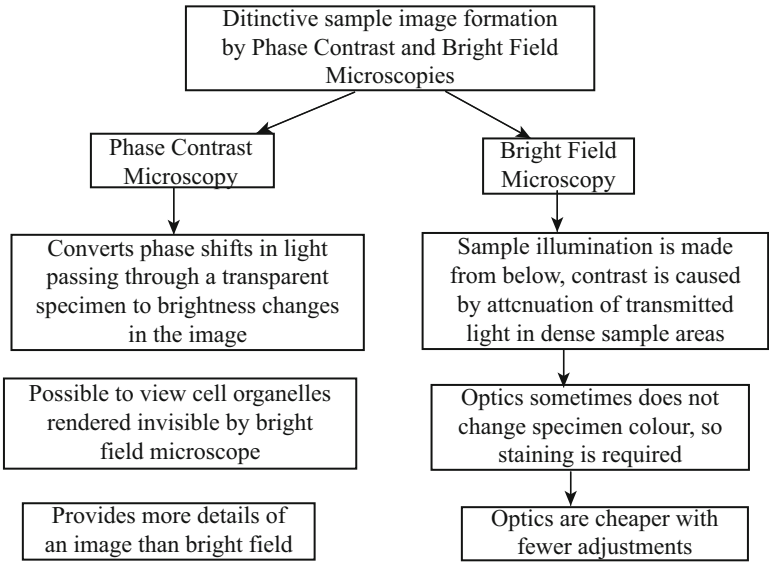


Fig. 10 Fundamental distinctions of phase contrast and bright-field microscopy

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.

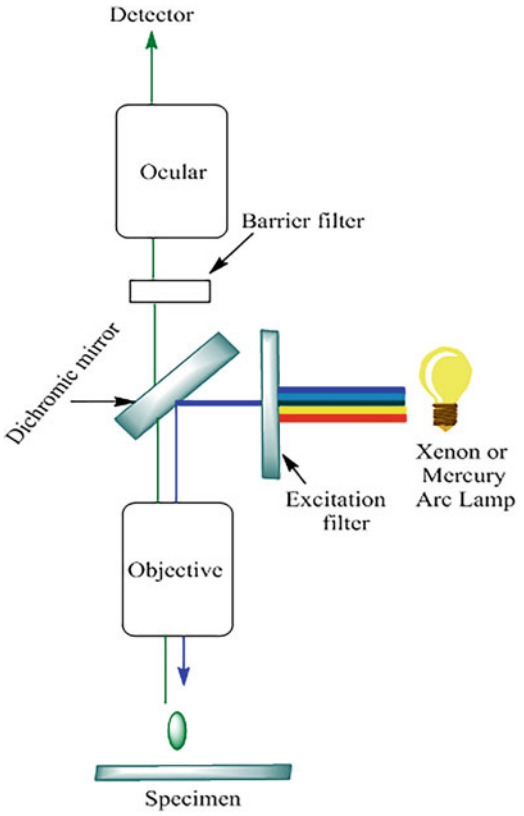


Fig. 11 Working configuration of a fluorescence microscope. Noteworthy distinctions from conventional microscopy include the elimination of out-of-focus light rays and enhanced sensitivity provided by laser scanning confocal microscope optical configuration

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).

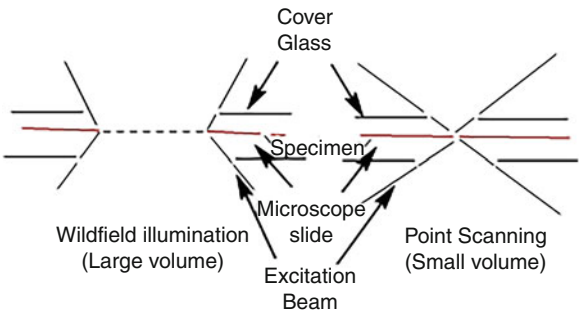


Fig. 12 The sample imaging distinctions in wide-field (electron microscopy) and point scanning (CM) modes

- 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

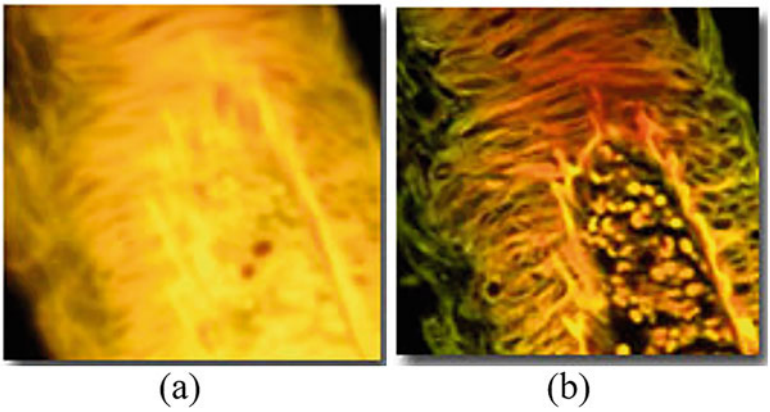


Fig. 13 Comparative images of fluorescence-stained human medulla using (**a**) traditional wide-field fluorescence and (**b**) laser scanning confocal microscope (retrieved from https://www.olympus-lifescience.com/en/microscope resource/primer/techniques/confocal/confocalintro/)

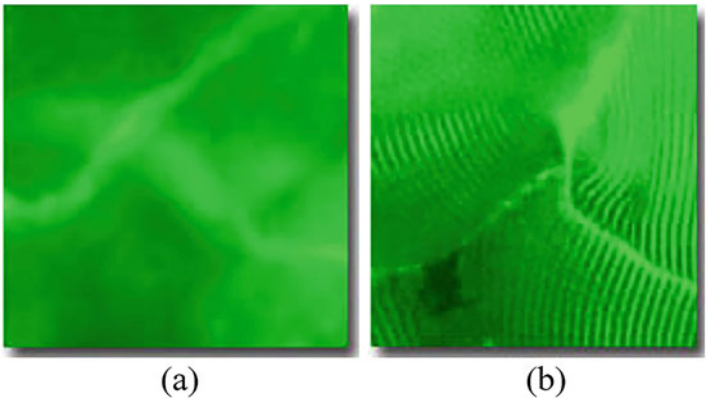


Fig. 14 Typical comparisons of whole rabbit muscle fiber topography analysis, viewed using (**a**) wide-field fluorescence and (**b**) laser scanning confocal microscope (retrieved from https://www.olympus-lifescience.com/en/microscope resource/primer/techniques/confocal/confocalintro/)

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

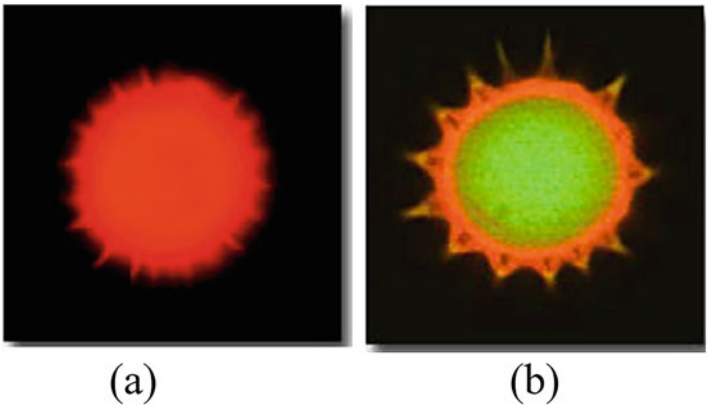


Fig. 15 Typical morphologies of the sunflower pollen grain, captured via (**a**) wide-field fluorescence and (**b**) laser scanning confocal microscope (retrieved from https://www.olympus-lifescience.com/en/microscope resource/primer/techniques/confocal/confocalintro/)

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.

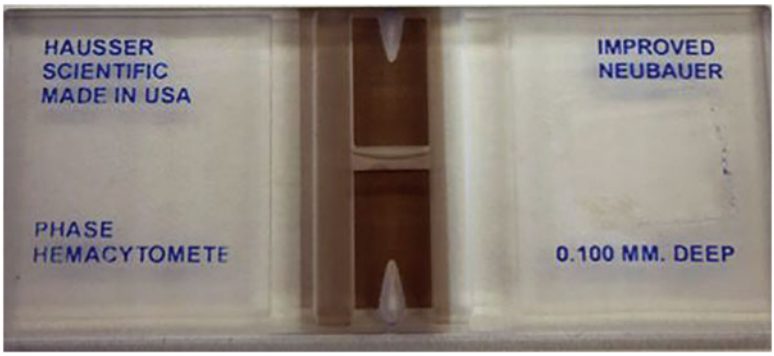
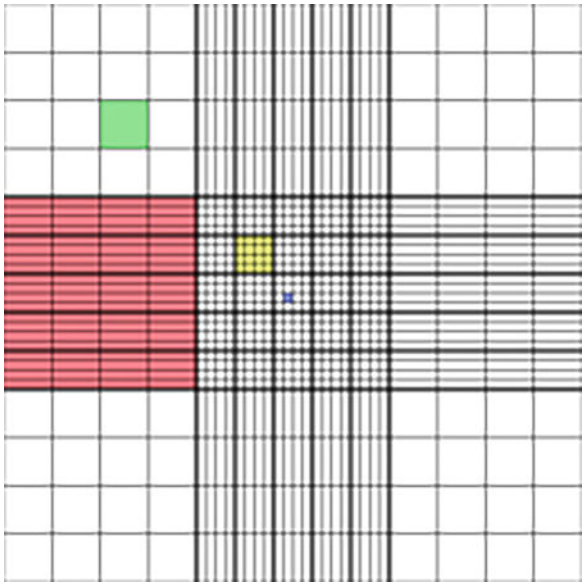


Fig. 16 Hemocytometer with H-shaped chamber in the middle

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.

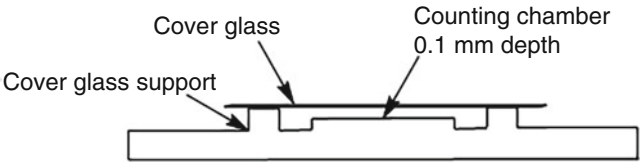


Fig. 18 Components of a hemocytometer, side-view coverage

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).**

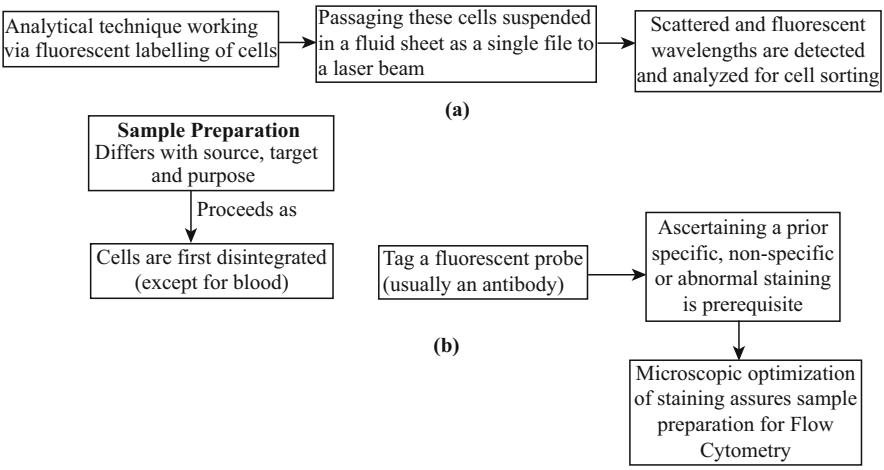


Fig. 19 Working principle (**a**) and sample preparation conditioning (**b**) in a flow cytometer. Sample preparation mandates a prior screening of residual staining impact

- 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.

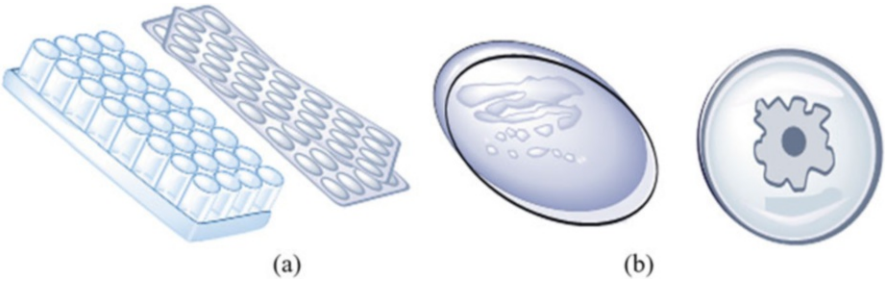


Fig. 20 Pictorial views of (**a**) culture plates and (**b**) Petri plates of different capacities

- 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.

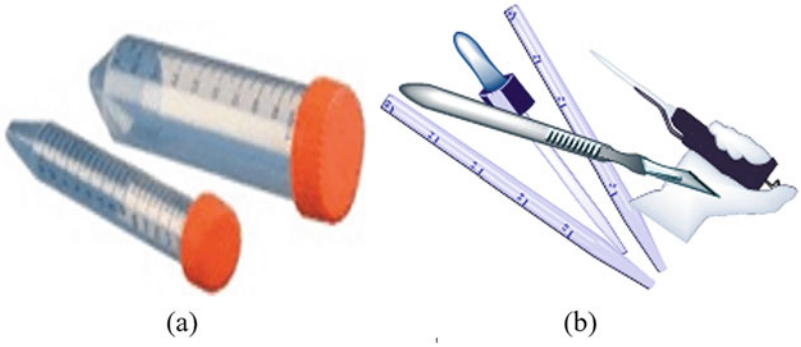


Fig. 21 Varying capacity (**a**) centrifuge tubes and (**b**) serological pipettes used for mammalian cell culture

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.

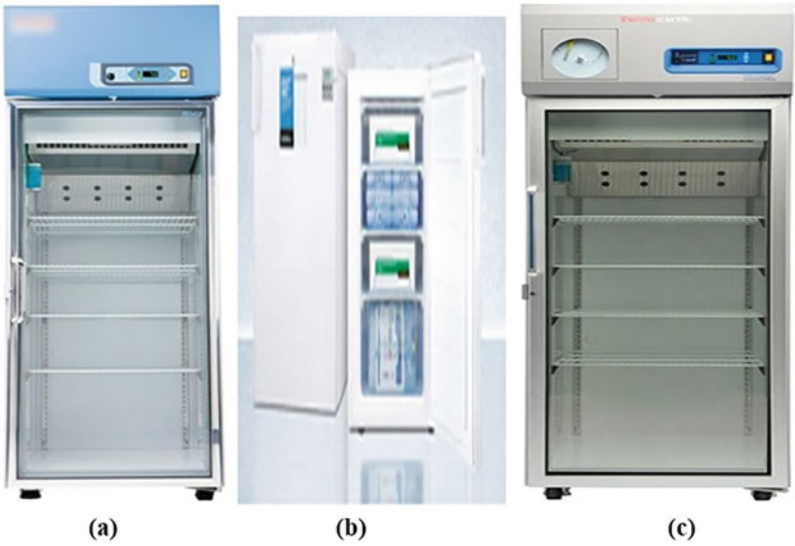


Fig. 26 Laboratory-scale refrigerators workable at (**a**) 4 °C, (**b**) −2 °C, and (**c**) −80 °C

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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