CELL BIOLOGY AND MEMBRANE BIOCHEMISTRY

Cell Division and Cell Signaling

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Cell Cycle and Cell Growth

Cell cycle and cell growth

Cell cycle is a highly ordered process that results in the duplication and transmission of genetic information from one generation to the next. The cell cycle is divided into various phases (Fig.1):

1. G1
2. S phase
3. G2
4. M phase

Interrupting every two mitotic phases, an interphase exists comprising of G1, S and G2 phases. Both extracellular and intracellular signals are responsible for governing the cells to progress through different stages of cell cycle. The G1 phase is associated with the cell growth. It is the preparatory phase for DNA synthesis. The S phase is devoted to DNA synthesis while G2 is another growth phase. The M phase comprises of the following stages sequentially:

1. Prophase: The replicated chromosomes condense and the mitotic spindle begins to assemble outside the nucleus.
2. Prometaphase: The membrane surrounding the nucleus (nuclear envelope) breaks down and allows the mitotic spindle to contact the chromosomes.
3. Metaphase: All the chromosomes are gathered at the center of the cell i.e. equatorial plate
4. Anaphase: The chromosomes are split apart and pulled towards opposite sides of the cell.
5. Telophase: The nuclear envelope reassembles around the two new sets of separated chromosomes to form two nuclei.
6. Cytokinesis: The last phase in which the other components of the cell, membranes, cytoskeleton, organelles, and soluble proteins, are distributed to the two daughter cells through a process called cytokinesis.

Once cytokinesis is completed, the cell has successfully gone through one turn of the cell cycle and produce two cells from a single precursor. For bacteria or yeast, which are single-celled organisms, this cell division will produce a new and complete organism. In a multicellular organism (like human beings), a fertilized single-celled egg requires many cell divisions to make a new individual. In either case it is the completion of the cell cycle that produces new organisms, a process that can go throughout life. The length of the cell cycle in multicellular organisms varies with cell types. In an adult human, for instance, adult nerve and skeletal muscle cells grow but do not divide. In contrast to this, epithelial cells are fast enough to divide twice a day.

Regulation and control of cell cycle

The regulation of the cell cycle must ensure that the events in each phase are complete before moving to the next. Thus checkpoints for monitoring the integrity of DNA are strategically placed in late G1 and at G2/M interface to prevent progression and propagation of mutated or damaged cells. G0 refers to cells that are quiescent (temporarily or permanently out of cycle). The normal cell is dependent on external stimuli (mitogens or growth factors) to move it out of

G0 and through the early part of G1. The time periods shown in Figure.1 are generic and only indicate the relative duration of each phase.

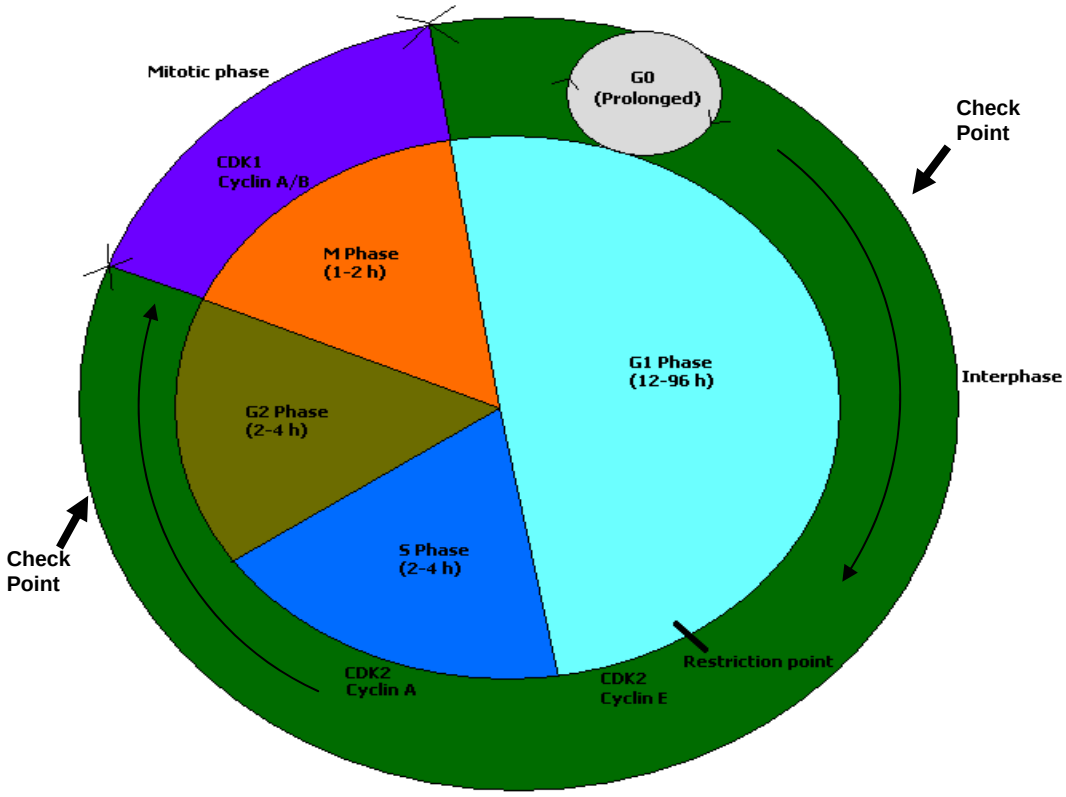

Fig.1: A diagram representing the various phases of generalized mammalian cell cycle

Cells have evolved many mechanisms that monitor various discrepancies occurring during their cell cycle, e.g. a fatal DNA damage. Because errors encoded in the genome may result in defective clones, close monitoring of the cell cycle for abnormal programming is mandatory. The best studied and probably the most important regulatory site is the checkpoint referred to as the "restriction point" (R) in the latter part of G1. An error occurring later in the cell cycle, in S or at G2/M, is recognized by checkpoint controls: depending upon the degree of damage, either the defect will be repaired or mitosis will be aborted.

In response to growth or mitotic signals, the cell moves out of G0 and through G1. In the absence of mitotic signaling, the cell may undergo differentiation, apoptosis, or enter the quiescent state (G0); the mechanisms responsible for taking the cell out of cycle into G0 or inducing differentiation are unclear.

Cyclin and cyclin –depenent kinases (CDKs) are the important molecules in the field of cell cycle regulation. Cyclins are the regulatory moieties of CDKs. Each cyclin makes a pair with the

specific CDK and performs distinct function in a particular phase of cell cycle. As the name suggests, the level of each cyclin independently increases or decreases within a particular phase of the cell cycle. Cyclin/CDK complexes phosphorylate specific protein substrates to move the cell through the cycle with activation of DNA synthesis (late G1 and S), and formation of the structural components associated with mitosis (late G2 and M). Well-delineated transitions between the cell cycle stages are controlled by synthesis and subsequent proteolytic degradation of cyclins.

The cycle begins in G1 with increased expression of the D cyclins (D1, D2, D3) upon external stimulation. The D cyclins associate with CDK4 and CDK6; formation of the cyclin/CDK complexes now get activated by the phosphorylation of CDKs by CDK Activating Kinase (CAK). The activated CDKs then phosphorylate the retinoblastoma (RB) protein. The RB protein plays crucial role in regulating G1 progression. The RB family members are “pocket proteins” that sequester the E2F transcription proteins; E2Fs are complexed with DNA—unphosphorylated or hypophosphorylated RB tightly binds E2F and inhibits transcription. Upon RB phosphorylation by CDK4/6, RB dissociates from E2F, allowing E2F to transcribe a number of responder genes required for passage through R (Fig. 2). RB is the gatekeeper of the cycle: hypophosphorylated RB guards the restriction point preventing cell cycle progression; hyperphosphorylation of RB is associated with release of E2F and passage through R. RB is maintained in its hyperphosphorylated state throughout the remainder of the cycle. As the cell progresses through late G1, there is increased expression of cyclin E. The cyclin E/CDK2 complex is required for the transition from G1 into S. Increased expression of cyclin A occurs at the G1/S transition and persists through S phase. With the binding of cyclin A to CDK2, DNA synthesis proceeds. In the latter part of S, cyclin A associates with CDK1. A checkpoint in G2 responds to DNA damage or incomplete DNA synthesis: progression into mitosis is delayed to allow DNA repair or the cycle is aborted. During M phase of cell cycle, cyclin B/ CDK1 complex gets activated to regulate the spindle fiber formation as well as reorganization of cytoskeleton.

Defective Cell Cycle in Cancer

Cancer cells tend to remain in cycle insensible to internal or external controls. Components of the cell cycle machinery are frequently altered in human cancers. The fundamental reason for such alterations is mutation in the genes involved in cell cycle regulation. The hallmark of the transformed state (cancerous state) is incompetent checkpoint control, resulting in aberrant responses to cellular damage. For example, damage to DNA or the spindle apparatus normally triggers cell cycle arrest or apoptosis, depending on the degree of damage and the cellular context. Cell cycle arrest most frequently occurs at the G1/S or G2/M boundaries. When checkpoint arrest control is compromised, initiation of S phase or mitosis occurs despite cellular damage, and the ensuing genetic instability may lead to the eventual emergence of a cancerous clone.

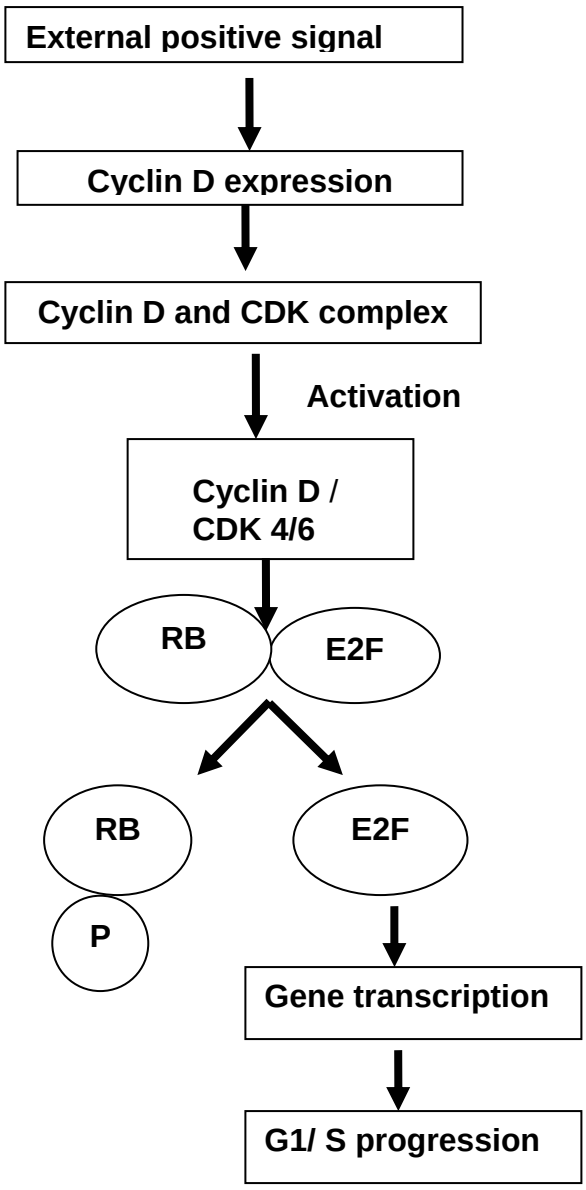

Fig. 2: Initial control mechanism to progress the cell through cell cycle

Cell and tissue culture techniques

Introduction

Study of any particular type of cell in its natural environment is generally not possible because of various constraints like growth of other organisms, temperature, pH etc. So, the necessity of cell culture came into picture that involves growing of cells in artificial condition, *in vitro*.

- **Tissue culture** – It is a very generic term involving the removal of cells, tissue or organs from animal/plant and subsequent incorporation into an artificial condition suitable for their growth. The liquid or semi liquid medium is supplied as the nutrients for their survival and growth.
- **Organ Culture** involves the culture of whole organs or intact organ fragments with the aim of studying their function and development. When the cells are removed from the organ/tissue (thus disrupting their normal relationship with neighboring cells) and cultured in artificial condition, it is called Cell Culture.

Primary Culture

The cells directly taken from the donor organism are called primary cells. When these cells are cultured in a suitable environment, they divide and grow. This process is called primary culture. This can be achieved by two ways- enzymatic treatment and explant culture. Enzymatic treatment (Trypsin or collagenase) onto the tissue fragments dissolves cement holding of the cells together, creating a suspension of single cells that is, thereafter, placed in the proper environment to grow and divide**. Explant Culture** involves the attachment of small piece of tissue with the substratum (glass or plastic vessel) bathed in culture medium. Gradually, individual cells move from tissue explants to the substratum and start growing and dividing.

Subculturing of cells

When primary cells grow and reach confluency, they need to be subcultured. Subculturing involves the enzymatic removal (breaking the peptide bonds attaching the cells to substratum) of the cells from the substratum, subdivision of the cell suspension formed and finally, placement of cell suspension into another vessel for further growth and division of the cells.

Secondary cells

Secondary cells are originally explanted from a donor organism. They divide and grow for many generations, after which they eventually senesce and die.

Immortalized cells

As the name indicates, immortalized cells continue to grow and divide indefinitely *in vitro* as long as favorable culture-conditions are maintained. These cells are having altered/transformed growth property, so, they are also called as transformed cells. They attain altered growth property by various mechanisms e.g., infection by transforming tumor virus or chromosomal changes. If these cells form tumors when injected into animal, they are considered to be Neoplastically Transformed.

Cell Culture Systems

There are two basic systems used for growing cells:

1. Suspension culture: cells divide and grow while floating in the culture medium
2. Monolayer culture: cells need the substratum for their growth and division. The substratum can be in the form of dishes, T-flask, bottles or multiple well plates.

The cells that can grow only when provided with substratum are considered to be **Anchorage Dependent**. These cells are generally derived from normal tissues. In contrast to this, the cells that are able to grow either attached to a substratum or floating free in suspension are meant to be **Anchorage Independent**. Transformed cells are anchorage independent cells.

Properties of cells in culture system

Property of the cells in culture varies with the change in the substratum, quality of cultured medium and incubation temperature. Basically, the difference in the properties of the cultured cells and their counterpart that grow *in vivo* arises from the dissociation of the cells from a three-dimensional geometry and their growth on a two-dimensional substratum. In the natural condition cells are grouped into tissue structure, so, cell- cell interactions are maintained. In cultured systems, cells are spread and lose interactions with each other. The cultured system also gets bereft of natural homeostasis, which is otherwise generated by neuro-endocrine system *in vivo*. The cultured cells depend largely on the glycolytic cycle for their energy requirement. Therefore, the cultured cell line may not necessarily be the true representative of their counterpart grown *in vivo*.

Biochemical markers can be used to determine if cells are still carrying on specialized function that they perform *in vivo* (e.g., liver cells secreting albumin, melanoma secreting melanin). Morphological or ultrastructural markers are also used as the tool (e.g., beating heart cells).

Uses of Cell Culture

Cell culture is extensively being used in cell and molecular biology. Some of the major areas where cell culture is currently playing crucial role are briefly described below:

Experimental Model System

Cell culture serves as a good model system for studying:

- The mechanism of basic cytological and biochemical processes taking place in any particular organism
- The host-pathogen interaction and its consequence
- Process of senescence
- The effect of drugs on cells

Cancer Biology

Cell culture has been a regular practice in the area of cancer research. The molecular mechanism by which normal cells convert into cancerous one can be studied by the use of various chemicals, radiations, viruses etc which could be useful in developing targeted anticancer therapy.

Screening of drug potency

Cell culture is used to identify the drug that is potent among all. Additionally, drug induced toxicity tests are performed on cultured cells to make sure that they are efficient and safe when applied on animals.

Biotechnological Industries

Cell cultures are routinely used in the industries to manufacture biotechnological products like:

- Monoclonal antibodies
- Vaccines
- Hormones
- Other useful proteins

Genetic engineering has been proven to be one of the most popular techniques to produce protein products at mass level in biotechnological industries. It involves the introduction of new genetic materials into the cultured cells that express protein of interest.

Gene Therapy

Cell culture has also shown its promising role in gene therapy. Gene therapy involves the replacement of missing or dysfunctional gene with the functional one. The cells can be grown for a while in culture and then replaced into the patient. The other approach for gene therapy involves transfection with genetically engineered virus carrying missing gene with the hope that this missing gene will be expressed in the patient cells.

Genetic counseling

Fetal cells are taken out from pregnant women, cultured and then tested for any chromosomal abnormalities by observing chromosomal banding patterns, karyotyping and other techniques. If any severe abnormality is found then parents are advised to refrain from giving birth to the child.

Cell culture in practice

Preservation and Storage

Cells are preserved in liquid Nitrogen either in liquid phase or in vapor phase. Freezing can have lethal effect on the cells due to the damage caused by ice crystals formed. Also, dehydration and change in the pH can occur upon freezing which cause damage to the cells. To minimize the effect of freezing, a cryo-protective agent (glycerol or DMSO) is used which lowers the freezing point. Additionally, isopropanol is used which allows the temperature to reduce gradually, about 1^{o} C per minute. The cells are slowly allowed to cool from room temperature to -80^{o} C so that water can move out of the cells before it freezes. Once the cells reach the temperature of -80^{o} C, they are immediately placed in the liquid nitrogen tank for storage. When needed, the cells are taken out and recovered after thawing at 37^{o} C.

Maintenance

Regular examination of cultured cells is necessary in order to check for any changes in the morphology or color of the medium. A proper record of name of the cell line, medium used, date of splitting/feeding, passage number etc should be maintained.

Feeding (medium change) is a necessary process for all types of cell lines to replace the exhausted and/or even toxic metabolites. Feeding to suspension culture is done by dilution into fresh medium while adherent cells are fed simply by replacing old medium with a new one.

When cells become semi-confluent, harvesting is done. The harvesting is required so that population density should not reach such a level, which suppresses the growth of the cells. If the cells are not harvested and are allowed to grow to a confluent state then there might be the probability for the long period of lag phase and some cells may never recover.

Harvesting can be achieved either mechanically or enzymatically. Mechanical harvesting involves physical removal of the cells from the substratum with the help of spatula. This may be highly disruptive causing death of the cells. So, viability is the limiting factor for mechanical harvesting. Enzymatic harvesting includes use of Trypsin, Collagenase or Pronase, in addition to EDTA, to detach the cells from the growth surface. This method can damage the cell surface by digesting exposed cell surface protein. The proteolytic reaction can be quickly terminated by the addition of complete medium containing serum.

Media and growth requirements

i. **Physiological parameters**
   - pH= 7.2-7.5
   - osmolarity of the medium must be maintained
   - Temperature= $37^{o}C$
   - Cells should be protected from direct light as it can produce toxic substances in the medium

ii. **Medium requirements:**
   - Na+, K+, Ca++, Mg++, Cl^{-}, PO_4^{3-}, HCO_3^{-} and CO_2
   - Fe, Zn, Se (Trace elements)
   - Glucose
   - 13 essential amino acids
   - Vitamins
   - Choline, Inositol

   Serum – It contains various growth factors and hormones. Also, it neutralizes the adverse effect of trypsin and other proteases on the cells.

   Antibiotics - although not required for cell growth, antibiotics are often used to control the growth of unwanted bacterial and fungal contaminants.

iii. **Feeding –** 2-3 times/ week.

Microscopy

Introduction

Microscopy began in 17th century, when people learned to make lenses which were used to produce magnifications that enabled them to observe microbes. Antony van Leeuwenhoek, was the first to report his observations with precise descriptions and drawings. He described protozoans found in rainwater as “very little animalcules” and gave the first documentation of a microscopic study. Microscopes are instruments designed to produce magnified visual or photographic images of objects too small to be seen with the naked eye. An ideal microscope must accomplish three tasks: produce a magnified image of the specimen, separate the details in the image, and render the details visible to the human eye or camera. Classification of microscopy is shown in Fig. 3.

These microscopic techniques have specialized applications discussed in detail later, though some limitations exist. In spite of the limitations, all these techniques reveal a unique and distinctive picture about the structure/ morphology of the sample.

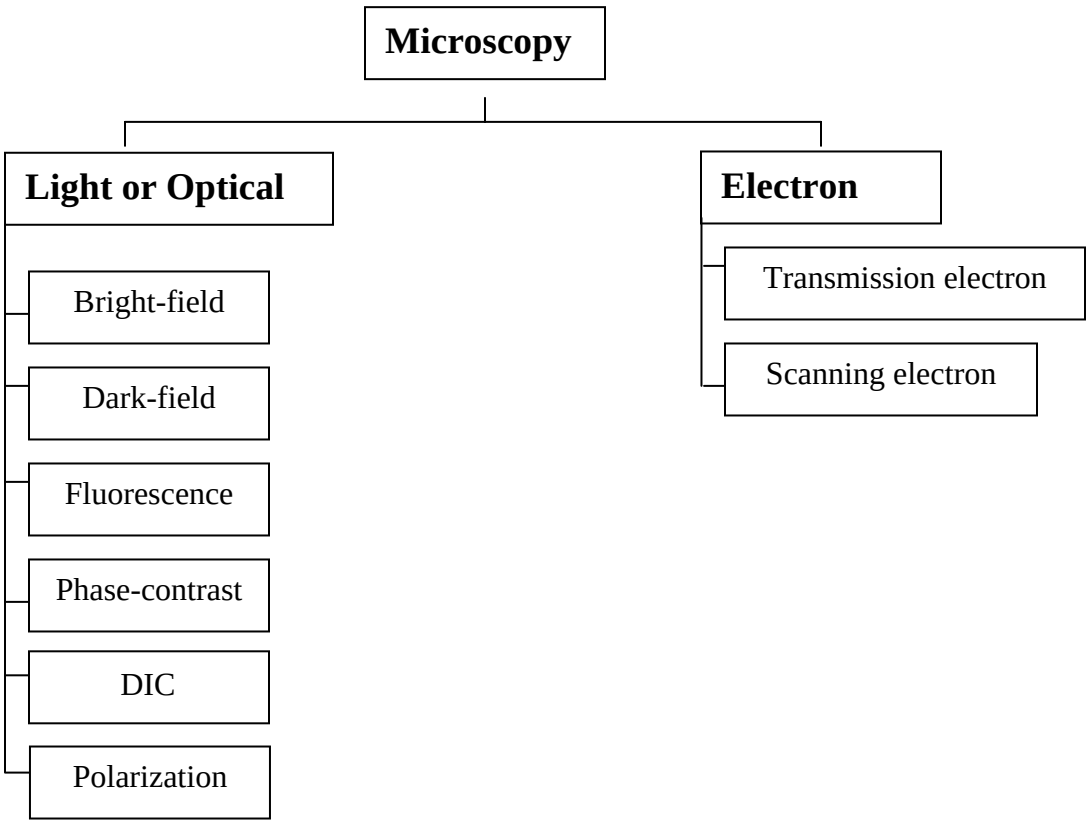

Fig. 3: Classification of Microscopy

Light Microscopy

It uses a system of optical lenses and light waves as a source of illumination.

Bright-field microscopy

Using a simple compound microscope and light waves as the source of illumination, in bright field microscopy, the microscopic field is brightly lit with microscopic sample appearing dark due to absorption of light. The main difficulty in bright-field microscopy is visualization of sample, as the native sample does not efficiently absorb light. Therefore, the sample is stained with a dye to increase its light-absorption ability and improve its visualization with greater contrast.

Bright-field microscopy is frequently used for histological studies of cells/tissues. Tissues obtained after surgery, biopsy or autopsy as well as blood films or smears are first fixed in formalin to avoid tissue/cell decay. The samples are prepared to make blocks after multiple baths of ethanol; followed by toluene, finally hot paraffin. During this 12 to 16 hour process, paraffin replaces the water and soft, wet tissues are turned into a hard block. This allows the sectioning of tissues into very thin (5 μM) sections using a microtome. These sections, thinner than the average cell, are then layered on a glass slide for staining. Stains are basically organic compounds called "dyes". Dyes can be classified into acid, basic or neutral. An acidic (anionic) dye shows a negative charge, basic (cationic) is a dye ion bearing a positive charge, whereas a neutral dye is a complex salt of a dye acid with a dye base. The basic principle of staining cells/cellular structures is ionic interactions. Apart from just ionic interactions, the staining process may involve ion-exchange reactions between the stain and active sites at the surface of or within the cell. Colored dye may replace other ions on cellular components, like, Na^+, K^+ etc. Stains and dyes are frequently used in cell biology to highlight structure in biological tissues for viewing. Stains help us to define and examine bulk tissues, cell populations, or organelles within individual cells. Staining protocols can be based on enzymatic activity present in the biological samples. Particular enzymes are localized to a particular organelle. Taking advantage of such a fact, the localization of enzymes can also be studied using bright-field microscopy. For example, 'osteoclasts' that are very rich in acid phosphatase, can be identified by using a dye namely, Naphthol AS-BI which upon catalysis by acid phosphatase is converted to a dark pink colored product that can be observed under the microscope.

Dark-field microscopy

This technique usually involves a simple compound microscope, but here the final image is brilliantly illuminated against a dark background. This effect is accomplished by using a special kind of condenser that transmits a hollow cone of light as a source of illumination. The most important application of dark-field microscopy is for examination of unstained microorganisms suspended in fluid-wet-mount and hanging drop preparations.

Wet preparations allow examination of organisms in its normal living conditions. A wet mount is made by placing a drop of fluid containing the organisms/cells onto a glass slide and covering the drop with a cover slip. To avoid evaporation of the fluid petroleum jelly or a similar material may be used to provide a seal between the slide and the cover slip. Usually a special slide with a circular concave depression is used for examining the wet preparations. A suspension of microbial specimen is placed on a cover slip, then inverted over the concave depression to

produce “hanging drop” of the specimen. The most important factor is control of light intensity to enhance visibility, as lack of stain make the cells less distinctly visible. This is done by adjusting the sub-stage condenser diaphragm.

Fluorescence Microscopy

Fluorescence microscopy is an extraordinarily sensitive method used to detect fluorescent molecules and minute material within the cell in the biological system. Fluorescent molecules absorb light at one wavelength and emit light at another, longer wavelength. When fluorescent molecules absorb a specific absorption wavelength for an electron in a given orbital, the electron rises to a higher energy level (the excited) state. Electrons in this state are unstable and will return to the ground state, releasing energy in the form of light and heat. This emission of light energy is the fluorescence. Because some energy is lost as heat, the emitted light contains less energy and therefore is a longer wavelength than the absorbed (or excitation) light. The main problem with fluorescence is to separate the fluorescence from incident light. To overcome this problem optical filters are used.

In fluorescence microscopy, a cell is stained with a dye (also called fluorochrome, it is a compound or part of a compound that imparts fluorescence to the sample) and the dye is illuminated with filtered light at the absorbing wavelength; the light emitted from the dye is viewed through a filter that allows only the emitted wavelength to be seen. The dye glows brightly against a dark background because only the emitted wavelength is allowed to reach the eyepieces or camera port of the microscope. Most microscopes are designed using epi-illumination. In epi-illumination excitation, light goes through the objective lens and illuminates the object. Light emitted from the specimen is collected by the same objective lens. Sometimes the fluorescent molecule itself is a direct stain or probe for specific structures. In other situations the fluorescent dye is bound to another non-fluorescent probe that recognizes specific structures. For example, the fluorescence molecule, rhodamine may be conjugated to phalloidin, which binds the filamentous actin. One important method to identify specific proteins is to couple fluorescent dyes to antibodies that bind very specifically to macromolecules in the cell. Sometimes the fluorescent molecule itself is a direct stain or probe for specific structures. Among the common fluorescence dyes are fluorescein, which emits green light when exited with blue light and rhodamine, which emits deep red fluorescence when excited by green-yellow light. The fluorescence microscopes are equipped with three fluorescent filter cubes, each containing specific barrier filters and a beam-splitting mirror. Fluorescence is naturally also seen in some proteins like GFP (Green Fluorescent protein), a protein produced by a jellyfish Aequorea, which fluoresces in the lower green portion of the visible spectrum. This protein is used in transfection studies of various genes and also in study of localization of various proteins.

Phase contrast microscopy (PCM)

Most detail of living cells is undetectable in bright field microscopy because there is too little contrast between structures with similar transparency and there is insufficient natural pigmentation so fixing and staining of cells is inevitable. Fixing and staining suffer a biggest drawback i.e., distortion or loss of certain cellular structures. Therefore, to examine the cells in their original state is to view them live without fixing and staining. Phase contrast microscope, invented by Frits Zernike in 1932, uses the difference in the refractive indices of cell constituents to visually differentiate them. Bending of light is a well known phenomenon when a beam of

light travels from one medium to the other. Highly refractive structures bend light to a much greater angle than do structures of low refractive index. The same properties that cause the light to bend also delay the passage of light by a quarter of a wavelength or so. In other terms, in phase contrast microscopy, light passing through a transparent part of the specimen travels slower and, due to this is shifted compared to the uninfluenced light. The difference in phase is not visible to the human eye. However, the change in phase can be increased to half a wavelength by a transparent phase-plate in the microscope and thereby causing a difference in brightness. This makes the transparent object shine in contrast to its surroundings. Phase contrast microscope has made it possible to study living cells and their normal processes such as mobility and cell division. Phase contrast microscopy is preferred at higher magnifications and provides an edge over normal bright-field microscopy.

Differential interference contrast microscopy (DIC)

Nomarski *differential interference contrast microscopy* (DIC) is based on the interference between two very closely located points in the object. The beam passing through the specimen is split by a birefringent plate (a modified Wollaston prism). The image is a gradient of the phase difference between these two adjacent points. Mathematically, the contrast denotes the path differences with respect to distance. This technique provides a greater resolution than any other light microscope. Furthermore, objects above and below the plane of focus are excluded from the image, thereby essentially providing an optical section. Nomarski optics is ideally suited for observing objects with well-defined boundaries, such as fibers or condensed chromosomes. It provides a higher contrast than the conventional phase contrast microscopy.

Polarization Microscopy

Form birefringence is a phenomenon in which plane polarized light is passed through the structure only if the plane of polarization is parallel to the long axis of the particles comprising the structure. This phenomenon is generally shown by the structure, whose particles are organized in a parallel array or a stacked disc embedded in a medium having refractive index different from that of the structure. This *form birefringence* is easily observed in cellular material using polarization microscope. This microscope plays an important role in determining the orientation of the particle. Also, when staining of the particles is very difficult and when their concentration or refractive index is too low, polarization microscopy has been proved to be the only rescue.

Electron Microscopy

It uses a high-voltage electron beam to produce the image of the specimen, using a set of electromagnetic lenses.

As the limit of resolution (the smallest distance by which two objects can be separated and still be distinguishable as two separated objects being inversely proportional to the wavelength of the source of illumination) depends on the wavelength of source of illumination, microscopes using light as a source of illumination have a limited resolution. Electron microscopy uses a beam of extremely short wavelength of the electron as a source of illumination. Therefore, a greater magnification is obtained. Electron microscopy is implemented in following two forms:

1. Transmission Electron Microscopy (TEM) and
2. Scanning Electron Microscopy (SEM)

Transmission Electron Microscopy (TEM)

The electron gun in TEM comprises a filament, a so-called Wehnelt cylinder and an anode. These three together form a triode gun, which is a very stable source of electrons. The tungsten filament is hairpin-shaped and heated to about 2700ºC. By applying a very high positive potential difference between the filament and the anode, electrons are extracted from the electron cloud around the filament and accelerated towards the anode. The anode has a hole in it so that an electron beam in which the electrons are traveling at several hundred thousand kilometers per second emerges at the other side. The Wehnelt cylinder, which is at a different potential, bunches the electrons into a finely focused point. The beam emerging from the gun is condensed into a nearly parallel beam at the specimen by the condenser lenses and, after passing through the specimen, projected as a magnified image of the specimen onto the fluorescent screen at the bottom of the column. If the specimen were not thin, the electrons would simply be stopped and no image would be formed. Specimens for the TEM are usually 0.5 μm or less thick. The higher the speed of the electrons, in other words, the higher the accelerating voltage in the gun, the thicker the specimen that can be studied.

Electromagnetic lenses: When an electrical current is passed through the coils (C), an electromagnetic field is created between the pole pieces (P), which form a gap in the magnetic circuit. By varying the current through the coils, the magnification of the lens can be varied. This is the essential difference between the magnetic lens and the glass lens. Otherwise they behave in the same way and have the same types of aberration: spherical aberration (the magnification in the centre of the lens differs from that at the edges), chromatic aberration (the magnification of the lens varies with the wavelength of the electrons in the beam) and astigmatism (a circle in the specimen becomes an ellipse in the image).

The condenser system: The condenser lens system focuses the electron beam onto the specimen under investigation as much as necessary to suit the purpose. The objective lens produces an image of the specimen, which is then magnified by the remaining imaging lenses and projected onto the fluorescent screen.

Specimen preparation: A TEM can be used in any branch of science and technology where it is desired to study the internal structure of specimens down to the atomic level. It must be possible to make the specimen stable and small enough (some 3 mm in diameter) to permit its introduction into the evacuated microscope column and thin enough (less than about 0.5 μm) to permit the passage of electrons. Every branch of research has its own specific methods of preparing the specimen for electron microscopy. In biology, for example, tissues are sometimes treated as follows: first, there is a chemical treatment to remove water and preserve the tissue as much as possible in its original state; it is then embedded in a hardening resin; after the resin has hardened, slices (sections) with an average thickness of 0.5 micrometers are cut with an instrument called an ultramicrotome equipped with a glass or diamond knife. The tiny sections thus obtained are placed on a specimen carrier – usually a 3 mm diameter copper specimen grid, which has been coated with a structureless carbon film 0.1 μm thick. Numerous other techniques

are available for use with electron microscopy, which extend its usefulness in characterizing cellular structures. Some are as follows:

1. Shadow casting
   Involves depositing an extremely thin layer of metal e.g. platinum, at an oblique angle on the specimen so that the specimen produces shadow on the uncoated side. This reveals a topographical representation at the surface of the specimen.

2. Negative staining
   The outline of the specimen is stained by an electron-dense material such as phosphotungstic acid, which forms thick deposits in the crevices of the biological sample. Finer details of objects like viruses or bacteria can be seen with this technique.

3. Ultra thin sectioning
   Uses ultramicrotome to prepare thin slices of the specimen at different angles and at different levels. This helps in identifying intracellular structures of the cell and also their morphological features. Improvement in contrast of structures is possible though use of special electron-microscopic stains such as uranium and lanthanum salts.

4. Freeze-fracture and Freeze-Etching
   In freeze-fracture, cells are frozen using liquid nitrogen (-196ºC) in the presence of a cryoprotectant to prevent distortion because of ice-crystal formation and then the frozen block is cracked with a knife blade. The fracture plane often passes through the lipid bilayers of the cell, thereby exposing the interior of the cell membranes. The resulting fracture planes are shadowed with platinum, the organic material is dissolved away, the replica is floated of and viewed under electron microscope. It has been successfully used to demonstrate the distribution of proteins in the membranes of cells. It is used to visualize either the exterior or interior of cells.

   In freeze-etching, the cells are frozen extremely rapidly- using a specially designed device to slam the sample against a copper block cooled with liquid helium. The frozen block is cracked with a knife blade as described above. But here the ice level is lowered around the cells by sublimation of ice in a vacuum (freeze-drying). The parts of the cell are exposed by this etching and are observed under the electron microscope. This technique reveals the interior of the cell and helps us to understand the three-dimensional organization within the cell.

Scanning Electron Microscopy

SEM also uses a narrow beam of electrons, which rapidly scans over the surface of the specimen, causing the release of a shower of secondary electrons and other types of radiation from the specimen surface. The intensity of these secondary electrons depends on the surface shape and the chemical nature of the irradiated object. These secondary electrons are detected by a detector, which generates an electronic signal used to generate image on the computer screen.

The difference between a SEM and TEM is that in SEM the column is considerably shorter because there are only three lenses to focus the electrons onto a fine spot on the specimen; in addition there are no lenses below the specimen. The specimen chamber, on the other hand, is larger because the SEM technique does not impose any restriction on specimen size other than that set by the size of the specimen chamber. The beam is not static as in TEM; it is scanned line by line over the object. The accelerating voltage is also much lower than in TEM. The specimen preparation too is much simpler than TEM. Comparatively, SEM has a low resolving power than TEM, but has the advantage of revealing the 3-D surface structure of the object.

Scanning Tunneling Microscope or Electron Tunneling Microscopy

The scanning tunneling microscope (STM) is a type of electron microscope that shows three-dimensional images of a sample. In the STM, the structure of a surface is studied using a stylus that scans the surface at a fixed distance from it. The operation of a scanning tunneling microscope (STM) is based on the so-called tunneling current, which starts to flow when a sharp tip approaches a conducting surface at a distance of approximately one nanometer. The tip is mounted on a piezoelectric tube, which allows tiny movements by applying a voltage at its electrodes. Thereby, the electronics of the STM system control the tip position in such a way that the tunneling current and, hence, the tip-surface distance is kept constant, while at the same time scanning a small area of the sample surface. This movement is recorded and can be displayed as an image of the surface topography on computer. Under ideal circumstances, the individual atoms of a surface can be resolved and displayed. Use of scanning tunneling microscopy is mainly in contour maps of surfaces in physics but it is also possible to fix organic molecules on a surface and study their structures. For example, this technique has been used in the study of DNA molecules.

Drawbacks of electron microscopy

1. The samples are viewed under vacuum for which the sample should be completely dried and hence we cannot see cells in their live state,
2. The processing of samples like freezing etc, might damage its morphological charactersistics, &
3. Low penetration power of electron beam necessitates the use of thin sections of samples.

Suggested Readings

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