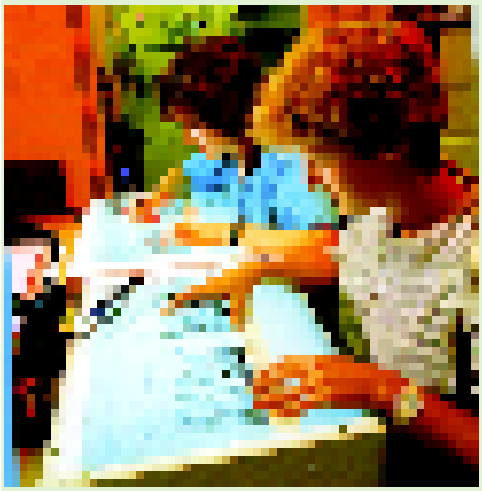
CHAPTER

2

Techniques in Cell Biology



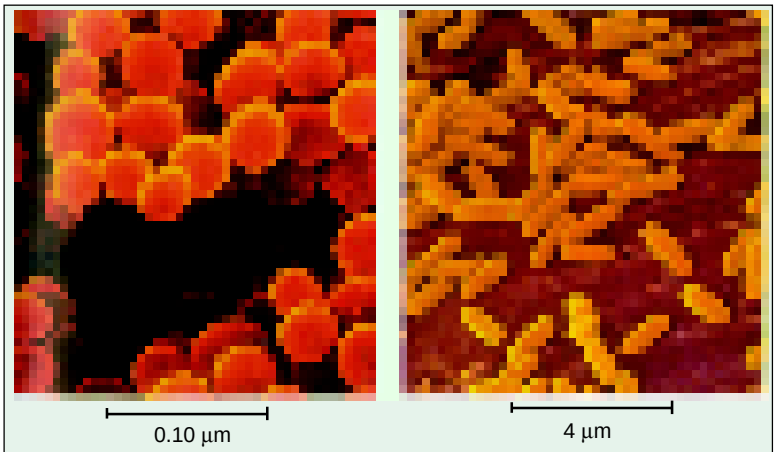
Work in a cytology laboratory where DNA, extracted from human cells, is analysed by techniques including electrophoresis and autoradiography.

Cells are tiny but complex bodies. It is difficult to see their structure; more difficult to understand their molecular composition and still difficult to find out the function of their various components. What one can learn about cells, depends on the tools at one's disposal and, in fact, major advances in cell biology have frequently taken place with the introduction of new tools and techniques to the study of cell. Thus, to gain divergent types of information regarding cell's structure, molecular organization and function, cell biologists have developed and employed various instruments and techniques. A basic knowledge of some of these methods is earnestly required.

MICROSCOPY

In the search for information about the structure and composition of cells, the cell biologists immediately face two limitations : the exceedingly small dimensions of cells and their component parts and the transparent nature of cells. The diameters of the majority of cells fall within a range of 0.2 and 50 µm. The human eyes have limited distinguishing or resolving power. The ability of an observational instrument such as a human eye or a microscope to reveal details of structure is expressed in terms of **limit of resolution** (*l*) which is defined as *the smallest distance that may separate two points on an object and still permit their observation as distinct separate points.* The un-

aided human eye under optimal conditions in green light (to which it is most sensitive) cannot distinguish between points less than about 0.1 mm or 100 μm apart. Structural details smaller than this, *e.g.*, cell, is unresolved unless some instrument capable of higher resolution is used. **Magnification**, *the increase in size of optical image over the size of the object being viewed*, is of no use unless the observational system can resolve the various parts of the structure being examined. Increased magnification without improved resolution results only in a large blurred image. The human eye has no power of magnification, so magnifying glasses may be used to magnify images up to about 10 times. A light compound microscope in which many lenses are combined together has a useful magnification of about 1,500 times.



Microscopes magnify microorganisms manifold and help us to determine their shape and structure like spherical shaped cocci and rod-shaped, bacilli bacteria as shown here.

The limit of resolution (l) of any optical instrument (*i.e.*, eye or microscope) is given approximately by the Abbe's relationships :

$$\text{Resolution } (l) = \frac{\text{wavelength } (\lambda)}{\text{numerical aperture } (n \sin \alpha)}$$

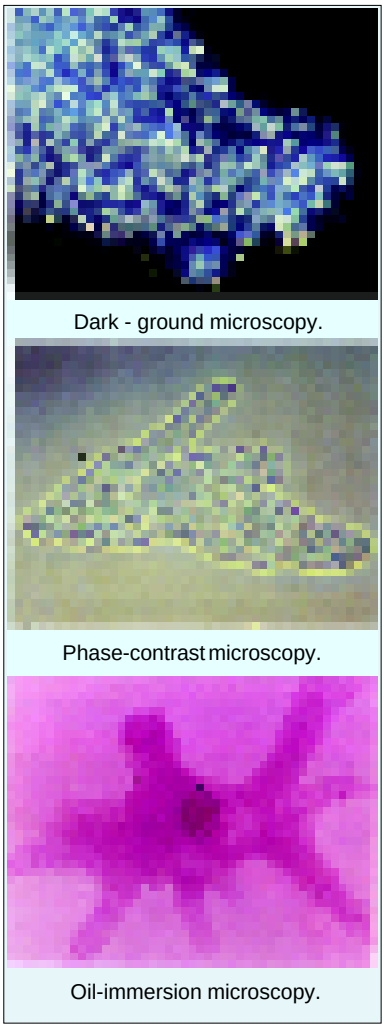
where λ (lambda) is the wavelength ("colour") of the illumination or radiation used to form the image, n is refractive index (a function of density) of the material (*i.e.*, mostly air or water) between the specimen and the first lens (or objective lens), and $\sin \alpha$ is sine of the semi-angle of aperture of the first lens as viewed from the specimen. The quantity "$n \sin \alpha$" is often called the **numerical aperture** (NA).

Abbe's relationships make it clear that high resolution in a microscope can only be achieved by manipulating a small number of variables: the wavelength of the illuminating radiation, the refractive index and the aperture. The **aperture** is limited to something less than 90° since that would have the lens and specimen in contact with one another. In fact, 85° is about the limit in good optical microscopes. Such angles require an excellent lens. In most cases, the aperture is less because the edges of the lens introduce distortions and so cannot be used. **Refractive index** is easy to alter, but only within narrow limits. It can be increased by using oils to fill the space between the specimen and the objective lens. Transparent immersion oils used in today's microscopes (*i.e.*, **oil immersion lens**) have n up to about 1.6. Still 1.6 is big improvement over air or water (n=1). In a microscope, the smallest detectable detail is equal to about one-half the **wavelength** of light with which it is observed. The smaller the object, the shorter the wavelength of light required. Hence, the wavelength of light is the area which has great chances of improvement. One can, for example, use ultraviolet light instead of visible light, thus, improving resolution as much as twofold. In order to do that however, special lenses (*e.g.*, of quartz) must be used since ordinary glass blocks much ultraviolet light. In such a microscope, called **ultraviolet microscope,** the eyes cannot be used to view the image directly, for they are insensitive to ultraviolet light. Lastly, a specimen cannot absorb light of wavelength below 0.3 μm.

Thus, a good light microscope, with a numerical aperture of 1.4 and using light of short wavelength (0.4 μm) will resolve two points at about 0.17 μm separations. By such a microscope though, one can see considerable details in most cells, there is also a great deal that cannot be seen. For instance, ribosomes and chromatin threads of nucleus are about 0.02 μm in diameter and quite invisible to the light microscope. For them electron microscope is used. In cell biological studies, the following two types of microscopes are most extensively used :

Light Microscopy

The **compound light microscope** uses visible light for illuminating the object and contains glass lenses that magnify the image of the object and focus the light on the retina of the observer's eye. It consists of two lenses, one at each end of a hollow tube. The lens closer to eye is called **ocular lens** or **eyepiece** and the lens closer to the object being viewed is called **objective lens** (Fig. 2.1). Usually objective lenses of various magnifying powers are mounted on a revolving turret at the lower end of the tube. The object, supported by a glass slide under the objective lens, is illuminated by light beneath it. In ordinary microscopes light is reflected on the object by a mirror having concave and plane surfaces. In some microscopes, a third lens, called **condenser lens**, is located between the object and the light source and serve to focus the light on the object.



Dark - ground microscopy.

Phase-contrast microscopy.

Oil-immersion microscopy.

In order to make full use of available resolving power of the compound light microscopes, special techniques have been designed to improve contrast. Certain improved types of light microscopes are of the following types :

1. Dark field microscope or Ultramicroscope. This type of light microscope is particularly useful for viewing suspensions of bacteria. In it, the object is viewed only with oblique rays and since one sees only those light rays that are scattered from objects, the images appear bright on a black background. The process is akin to seeing dust particles floating in a sunbeam.

2. Phase contrast microscope. This type of light microscope takes advantage of the fact that different parts of a cell have different densities and, hence, different refractive indices. Regions where the refractive index is changing they tend to bend light rays. In phase contrast microscope these bent rays are used to form patterns of destructive interference, yielding sharp contrasts. This technique is widely used to observe unstained and living cells (especially in mitotically dividing cultured cells).

The **interference microscope** is based on the principle of the phase contrast microscope and permits detection of small, continuous changes in refractive index. The variations of phase can be transformed into such vivid colour changes that a living cell looks like a stained preparation.

3. Polarization microscope. This type of light microscope is useful mainly for viewing highly ordered objects such as crystals or bundles of parallel filaments (*i.e.*, microtubules of mitotic spindle.) It is based on the behaviour of certain cellular materials when they are observed with polarized light. If the material is **isotropic**, polarized light is propagated through it with the same velocity, independent

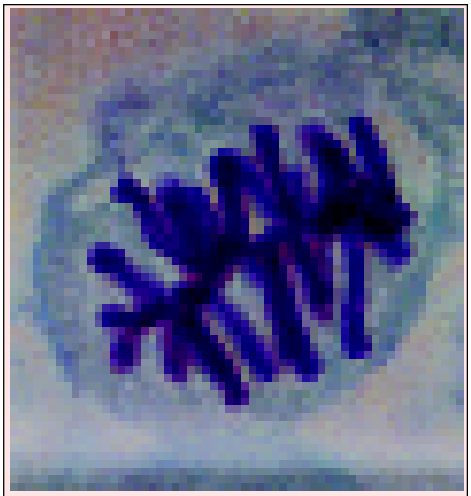
of the impinging direction. Such substances are characterized by having the same **index of refraction** in all directions. On the other hand, in an **anisotropic material** the velocity of propagation of polarized light varies. Such a material is also called **birefringent** because it presents two different indices of refraction corresponding to the respective different velocities of transmission. In a polarizing microscope, the specimen is placed between two closed polarizers and visible birefringent portions of the sample act like polarizing films and, hence, these portions of the sample are seen as bright objects on dark background.

Methods of Sample Preparation for Light Microscopy

Cells are transparent and optically homogeneous: so either they are viewed as such by instruments such as phase contrast microscope or to produce necessary contrast, the cells are passed through various steps of slide preparation such as killing, fixation, dehydration, embedding, sectioning, staining and mounting.

Thus, superior specimens for microscopic examination can be obtained by **killing** the cells and coagulating or **fixing** the protoplasm by preservatives, called **fixatives** such as alcohols, formaldehyde, mercuric chloride, picric acid, acetic acid and mixture of these. The process of fixation involves the following events — (1) The proteins and other macromolecules are precipitated. (2) The intracellular hydrolytic enzymes are denatured, preventing autolysis. (3) Cross links are formed between macromolecules, making the preparation more stable and minimizing shrinkage upon drying. (4) Substances are introduced which prevent attack by microorganisms. (5) The tissues become stiffer, making their sectioning easier. (6) The affinity of the tissue for dyes (stains) is increased.

Fixation is generally followed by **dehydration** (*i.e.*, gradual removal of water vapours from the tissue) by the organic solvents such as ethanol. The dehydrated specimens are **embedded** *i.e.*, they are infiltrated with molten paraffin which hardens upon cooling and provides enough support to allow thin sections to be cut with a **microtome**. By the microtome, serial sections, 5 to 10μm thick can be cut and placed on slides in the order of cutting and permitting a sequence of specimens for observation. These sections are stained with a non-vital-stain to increase the contrast.



The Feuglen stain - This staining is specific for DNA.

Stains are the chemicals that can selectively attach to particular molecules of particular cellular structures and make them stand out from other parts of the cell. The non-vital stains fall into two main classses: **acid stains** such as eosin, orange G, aniline blue and fast green, all of which combine with basic molecules such as proteins of the fixed cells; and **basic stains** such as methylene blue, crystal violet, haematoxylin, basic fuchsin, etc., all of which combine with nucleic acids and other acidic molecules of the fixed cells. The cellular structures that stain with acid stains are called acidophilic and those that stain with basic dyes are called **basophilic**.

In addition, there are certain specific stains, called **cytochemical stains** that bind selectively to some specific groups of cellular macromolecules such as proteins, nucleic acids, polysaccharides and lipids. For example, Millon reaction, diazonium reaction and Naphthol Yellow 5 stain are used for the proteins; alkaline fast green is used for histone (basic protein); Feulgen reaction (using Schiff's reagent) is used for DNA; methyl green-pyronine stain (Unna-Pappenheim stain) is used in distin-

guishing between DNA and RNA and it stains DNA green and RNA red; acetocarmine and acetoorcein stains are used to stain chromosomes of dividing cells; periodic acid-Schiff (PAS) reaction is used for the demonstration of polysaccharide materials such as starch, cellulose, hemicellulose, and pectin in the plant cells and mucoproteins (glycoproteins), hyaluronic acid and chitin in animal cells; and fat soluble dyes such as Sudan Red and Sudan Black B are used for the lipids. The Sudan Black B is a specific stain for phospholipids and is used to stain Golgi apparatus.

Vital stains selectively stain the intracellular structures of living cells without serious alteration of cellular metabolism and function. For example, Janus green B selectively stains mitochondria; neutral red stains plant vacuoles and methylene blue stains Golgi apparatus and also nuclear chromatin of dividing cells.

All these steps often are time-consuming and cause artifacts in the cells. Hence, when speed is important and specimen is required for electron microscopy or for histochemical analysis, paraffin embedding may be replaced by fixation by freeze drying. **Freeze drying** is a method that avoids denaturation of enzymes and is particularly useful for histochemical staining. Tissue is frozen rapidly by plunging its small portions into liquid carbon dioxide or liquid nitrogen and, thus, required rigidity for sectioning by the **freezing microtome** is obtained. Frozen sections are stained and are dehydrated at low temperature (–30 to –40^{0}C) in a high vacuum. At such low temperature ice crystals are of minimum size and few distortions or artifacts arise. Chemical composition and physical structure are maintained with little change. Another advantage is that fixation is rapid enough to arrest some cellular functions at their critical junctures which can then be observed and compared.

Electron Microscopy

The electron microscopy (Fig. 2.1) uses the much shorter wavelengths of electrons to achieve resolution as low as 3 A^0, with a usual working range between 5 to 12 A^0. In the electron microscope electromagnetic coils (*i.e.*, magnetic "lenses") are used to control and focus a beam of electrons accelerated from a heated metal wire by high voltages, in the range of 20,000 to 100,000 volts (new instruments are being developed that use 1,000,000 volts). The wavelength of an electron depends on the magnitude of the voltage and may be 0.01 A^o or

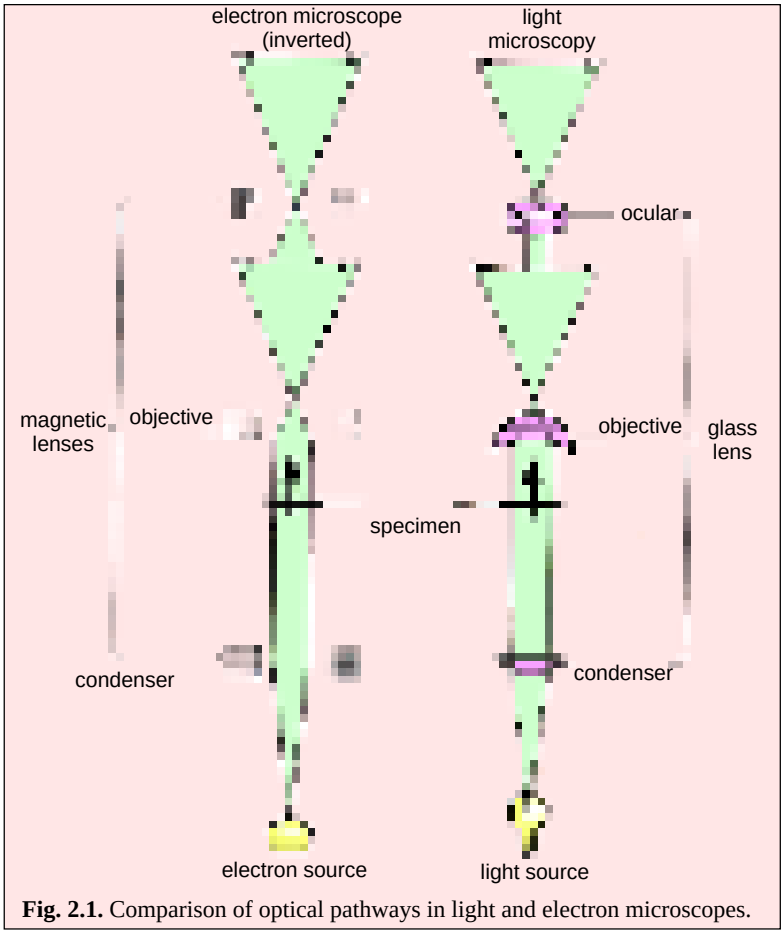
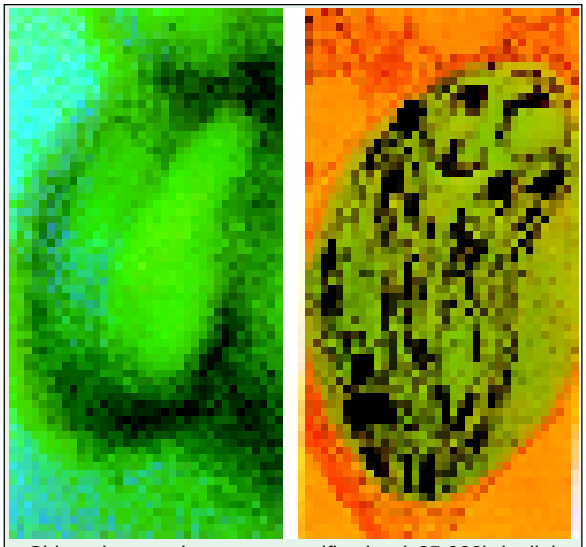


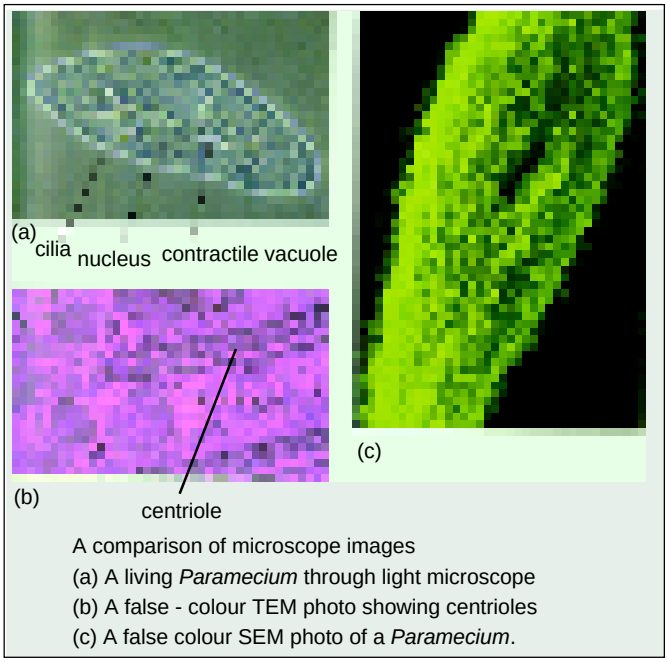
Fig. 2.1. Comparison of optical pathways in light and electron microscopes.



Chloroplasts at the same magnification (x25,000), by light microscopy (left), and by electron microscopy (right).

less. The electrons of the beam are scattered by a specimen placed in the path of the beam. Electrons that do manage to pass through the specimen are focused by an objective coil ('lens') and a final magnified image is produced by a projecter coil or 'lens'. The final image is viewed directly on the fluorescent screen or is recorded on photographic film to produce **electron micrograph**. This type of electron microscope is called **transmission electron microscope** (**TEM**).

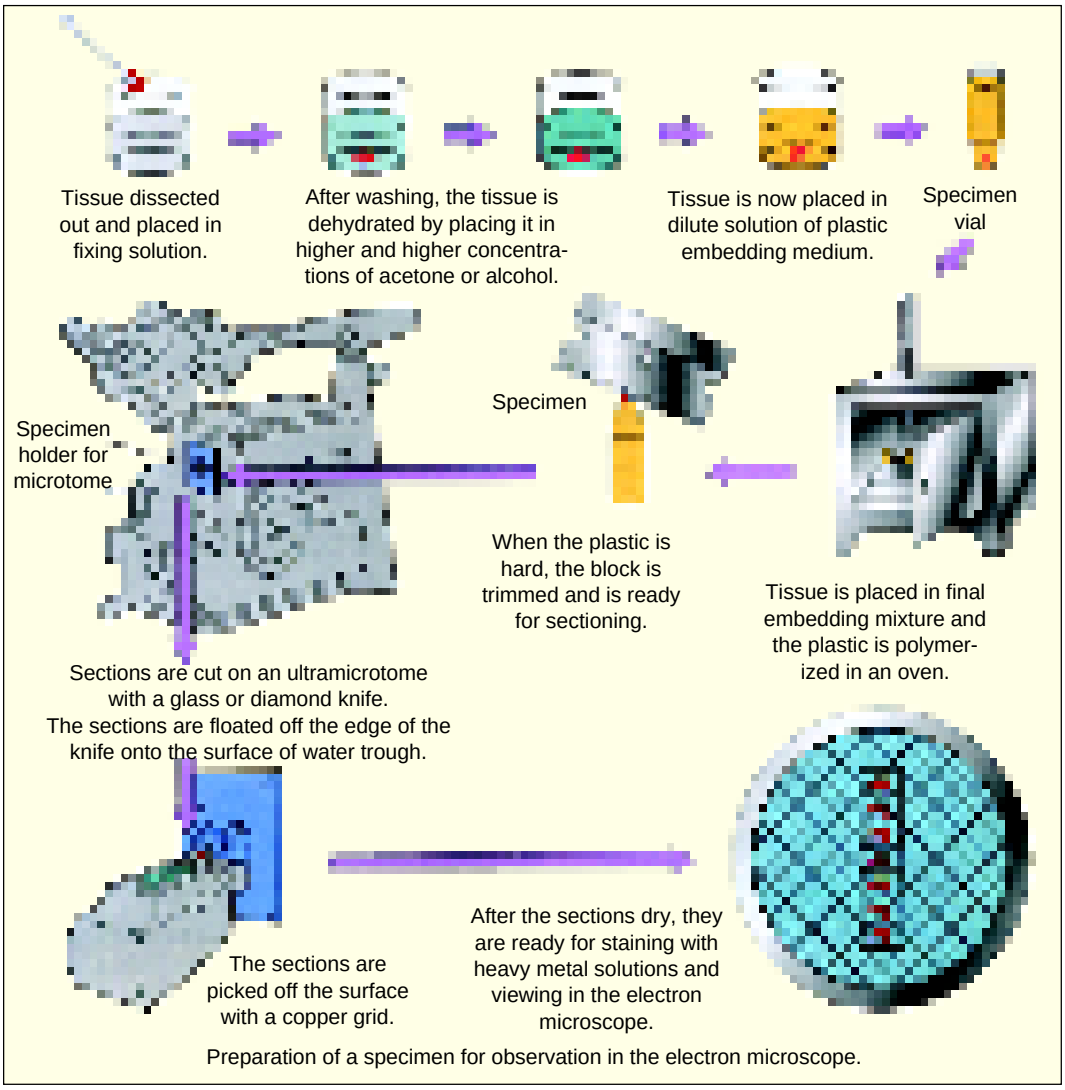
Unlike the compound light microscope, in which image formation depends primarily upon differences in light absorption, the electron mircroscope forms images as a result of differences in the way electrons are scattered by various regions of the object. Electrons have a very low penetrating power, that is, they are easily scattered by objects in their paths. The degree to which electrons are scattered is determined by the thickness and atomic density of the object: regions of high density(possessing atoms of high atomic number) scatter electrons more than regions of lesser density and consequently appear darker in the final image. Because electrons are scattered so easily, the specimen used in electron microscopy must be extremely thin (ultrathin, *i.e.*, 10 nm to 100 nm thick). If the sections were not extremely thin, most of the electrons would be scattered and a uniform dark image would result. Since, electrons are scattered even by gas molecules and so the electron beam must travel through the electron microscope in a very high vacuum and the samples must be completely dry and otherwise non-volatile. Thus, living cells which are wet cannot be viewed in electron microscope.



A comparison of microscope images
(a) A living *Paramecium* through light microscope
(b) A false - colour TEM photo showing centrioles
(c) A false colour SEM photo of a *Paramecium*.

Methods of Sample Preparation for Transmission Electron Microscopy

The standard procedure for the preparation of specimen for TEM entails fixation, dehydration, staining and sectioning similar to light microscopy (Table 2-1.). However, the most significant difference being the need for ultra-thin sections. Following techniques of sample preparation are generally used for different types of methods of studying ultrastructure of the cell:



Preparation of a specimen for observation in the electron microscope.

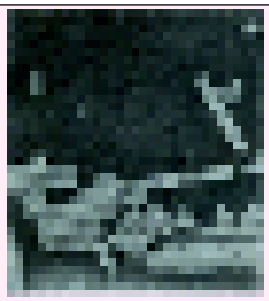
Table 2-1. Comparison between light and electron microscopy.

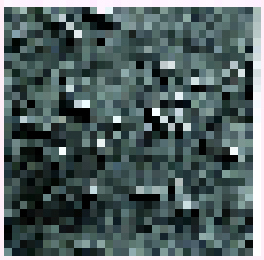
Steps	Electron Microscopy	Light Microscopy
Fixation	Osmium tetroxide, potassium permanganate, formalin, glutaraldehyde.	Bouin's solution; formalin; Zenker's fluid.
Dehydration	Increasing concentration of ethanol (or acetone) followed by propylene oxide.	Increasing concentrations of ethanol followed by benzene.
Embedding	Araldite: Vestoplaw, Epan 812; Maraglas; Durcopan.	Paraffin.
Sectioning	Usually 10–100 nm thick sections cut with a glass or diamond knife on an ultramicrotome.	Usually 6µm thick sections cut with a razor blade on a microtome.

Steps	Electron Microscopy	Light Microscopy
Mounting	On a perforated metal disc (grid) usually covered with formvar or paralodian.	On a glass slide with an egg albumin adhesive. Deparaffinized in xylol for staining.
Staining	With salts of heavy metals such as lead acetate, lead citrate, lead hydroxide, uranyl acetate; phosphotungstic acid.	Selective chromatic stains (as haematoxylin and eosin), dehydrated in ethanol series, cleared in xylol and mounted for viewing in Canada balsam or Permount.
Viewing	Grid is placed between the condenser and objective lenses in a vacuum and the image is viewed on a phosphorescent screen.	Slide is placed between the condenser and objective lenses and the image viewed in the ocular lens.

1. Monolayer technique. Macromolecules such as DNA and RNA are studied by monolayer technique in which the macromolecules are extended on the air-water interface before being collected on a film.

2. Thin sectioning. This method uses a cutting device known as **ultramicrotome** to remove ultrathin (*i.e.*, 10 nm to 100 nm thick) sections from the specimen. To withstand the passage of ultrafine diamond or glass knife without tearing, the specimen is first embedded in a hard plastic such as, epoxy resin (Table 2-1). The resin is allowed to penetrate the sample before it is polymerized. Sections are floated from the knife of ultramicrotome onto the surface of water and picked up by touching them with a fine wire mesh or small circular copper grid (*i.e.*, small discs perforated with numerous openings). Prior to its use mesh or grid is coated with a thin monolayer film (7.5 to 15nm thick) of plastic (such as formvar or collodion) or carbon to provide a support to the sections (or sample) (Fig.2.2). The specimen is visualized through the holes of screen.





Examples of negatively stained and metal shadowed specimens.

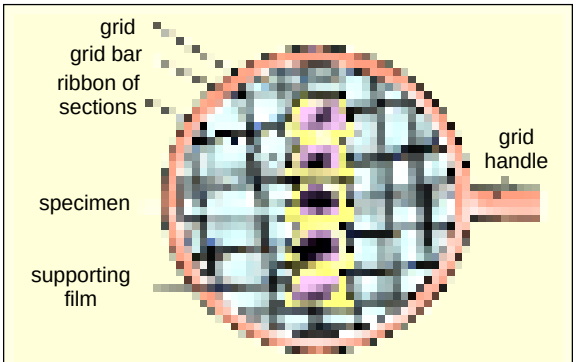


Fig. 2.2. Appearance of a ribbon of ultrathin sections on the grid (after Sheeler and Bianchi, 1987).

Sections to be examined with the electron microscope are generally not stained (since no colours are seen with the electron microscope). However, contrast may be improved by "poststaining" with **electron stains** or electron-dense materials such as urynyl acetate, urynyl citrate, lead citrate, osmium tetroxide, etc. The method of thin sectioning is used to study morphology of cell.

3. Negative staining. This technique is used to study small particles such as viruses or macromolecules. Here, the specimen is embedded in a droplet of electron dense material, such as, phosphotungstic acid ($H_3PW_{12}O_{40}$). The electron stain penetrates into all the

empty spaces (*i.e.*, openings and crevices) between the macromolecules. The spaces appear well defined in negative contrast. The portions of specimen that exclude stain transmit electrons readily, so their images can be seen.

4. Shadow casting or heavy metal shadowing. This technique is used to study three-dimensional appearance of viruses and certain macromolecules such as DNA molecules and collagen fibres. It involves placing of specimen in an evacuated chamber and evaporating at an angle, a heavy metal such as chromium, palladium, platinum or uranium from a filament of incandescent tungsten. The vapour of heavy metal is deposited on one side of the surface of the elevated particles; on the other side a shadow forms, the length of which permits determination of the height of the particle. In such a specimen, during electron microscopy, the electrons pass readily through the area of light metal content, less readily through the plane on which the particle sits, and are scattered more severely by the side of the particle on which metal has accumulated. Thus, by shadow casting, shape and profile of a particle can be discerned.

5. Tracers. Several biological processes such as pinocytosis, phagocytosis and transport of molecules across plasma membrane can be studied by the use of appropriate tracers (*e.g.*,gold, mercuric sulphide, iron oxide, etc.). These tracers are detected by their electron opacity. An ideal tracer should be non-toxic, physiologically inert, composed of small-sized particles of uniform and known size and preserved *in situ* during the processing of the tissue.

6. Freeze-fracture. This technique is used to study the molecular arrangement in the plasma membrane and other cellular membranes. It is carried out by rapidly cooling or freezing the sample (**cryofixation**) and then **fracturing** (cracking) it in a vacuum while it is still at —100ºC. The knife does not cut cleanly under those conditions, but tends to **fracture** (crack) the specimen along the lines of natural weakness, such as the middle of a membrane that runs parallel to the cut (Fig. 2.3). After fracture, the sample is left in the vacuum long enough to allow some water to evaporate from the exposed surfaces, a process called **freeze etching**. The exposed face is then shadowed with electron-dense combination of carbon and metal such as platinum to provide the necessary contrast, after which organic material (*i.e.*, the specimen itself) is removed by acids to leave a metal **replica** for examination in the electron microscope. Replica reveals a natural-looking representation of the surface of the freeze-etched object and is the only way of seeing membrane interior and certain other features of the cells.

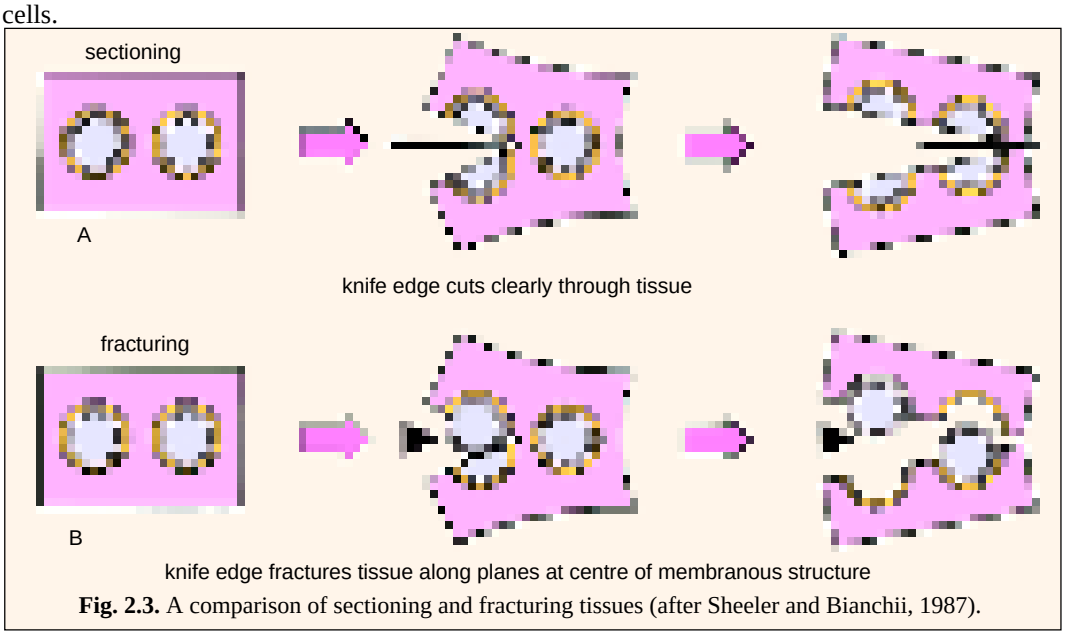
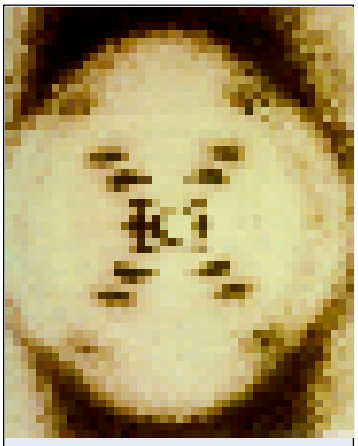


Fig. 2.3. A comparison of sectioning and fracturing tissues (after Sheeler and Bianchii, 1987).



The X-ray diffraction pattern of DNA.

7. Whole mounts. They are often used to examine chromosomes and other relatively thick objects that can be isolated free of debris. In these methods, the specimen is neither sectioned nor stained. Thick areas will scatter electrons more strongly than thin areas, providing enough contrast to form an image.

Scanning transmission electron microscopy (STEM). This electron microscope has less resolution power than the TEM (*i.e.*, about 200 A^0), yet is a very effective tool to study the surface topography of a specimen. In this instrument a narrow beam of electrons is scanned rapidly over a specimen and a three-dimensional image resulting from differential scattering of electrons by different parts of the surface of specimen is recorded on a cathode-ray oscilloscope and a photographic emulsion.

X- RAY DIFFRACTION ANALYSIS

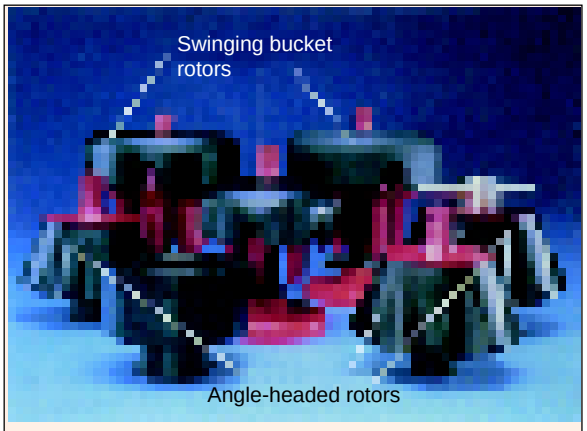
This technique is used to analyze three-dimensional (tertiary) structure of DNA molecule and a variety of proteins such as myoglobin, haemoglobin, collagen, myelin sheath of nerve cells, myofibrils of striated muscles, etc. This method depends on the fact that X-rays are scattered or diffracted by the atoms of a substance. If the material has an ordered crystalline atomic structure, the resulting X-ray diffraction pattern is also ordered and reflects the three-dimensional arrangement of atoms in crystal.

Scanning electron micrograph: the head and the mouthparts of a weevil.

CELL FRACTIONATION

Sometimes it becomes necessary to break up tissues and cells and to isolate various parts of the cell for structural or biochemical analysis. For this purpose, the technique of cell fractionation is employed. Cell fractionation method involves, essentially the homogenisation or destruction of cell boundaries by different mechanical or chemical procedures, followed by the separation of the subcellular fractions according to mass, surface and specific gravity by centrifuges.

Centrifuges in their various forms have become versatile tools of cell biology; they are used not only to characterize substances but to separate them. The **analytical ultracentrifuge** provides information concerning the mass and the shape of a molecule while **preparative ultracentri-**



A variety of rotors used in a preparative ultracentrifuge.

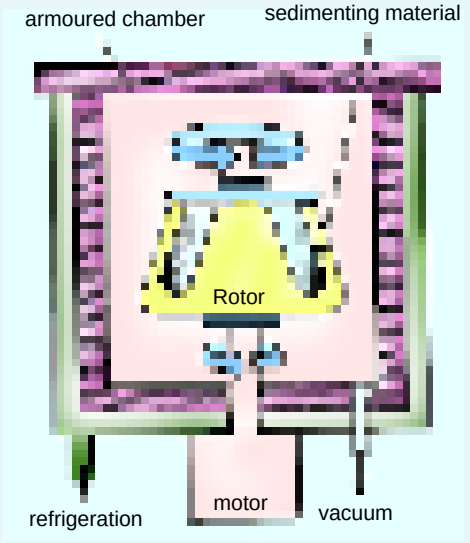


Fig. 2.4. The preparative ultracentrifuge. The sample (*i.e.*, homogenate) is contained in tubes that are inserted into a ring of cylindrical holes in a metal rotor. Rapid rotation of the rotor generates immense centrifugal forces, which cause particles in sample to sediment. The vacuum tends to reduce friction, preventing heating of the rotor and allowing the refrigeration system to maintain the sample at 4ºC (after Alberts *et al.*, 1989).

fuge (Fig. 2.4) permits one to use these parameters to separate molecular types. An ultracentrifuge differs from other centrifuges only in attaining higher rotor velocities (*i.e.*, up to about 70,000 revolutions per minute or rpm). In addition, the analytical ultracentrifuge contains an optical system, allowing one to observe changes in the solute distribution as they occur in the sample. The rotors of all ultracentrifuge spin in a vacuum in order to prevent heating from air friction.

In the cell fractionation, the cells are gently broken by grinding a small piece of tisssue in a homogeniser having a moving close-fitting glass or plastic pestle within a tube that contains a medium such as sucrose solution to preserve the cellular organelles (Fig. 2.5). The solution containing homogenised or disrupted cells, is called **homogenate**. The homogenate is subjected to differential centrifugations of increasing velocity. The method depends on the principle that particles of different weight or sizes move at different rates through a solution in a centrifugal field. At each step larger particles form a gelatinous pellet at the bottom of the tube leaving smaller particles in the supernatant solution. By decanting the supernatant and spinning it harder, the next fraction can be brought down. Ultimately one is left with a supernatant solution having only soluble, molecular-sized components. The residual solution is called **cytosol**. The different molecules of cytosol are isolated by a variety of biochemical techniques such as chromatography, dialysis and electrophoresis.

The technique of cell fractionation has been improved through the use of **density gradient centrifugation.** In this method, centrifuge tube is loaded with layers of solution of varying densities of either sucrose, heavy water, cesium chloride or albumin, in a gradient from top to bottom. Once the gradient is formed, the homogenate is layered on the top and centrifuged until the particles reach equilibrium with the gradient. For this reason, this type of separation is called **equilibrium density** or **isopynic centrifugation** (**King**, 1986).

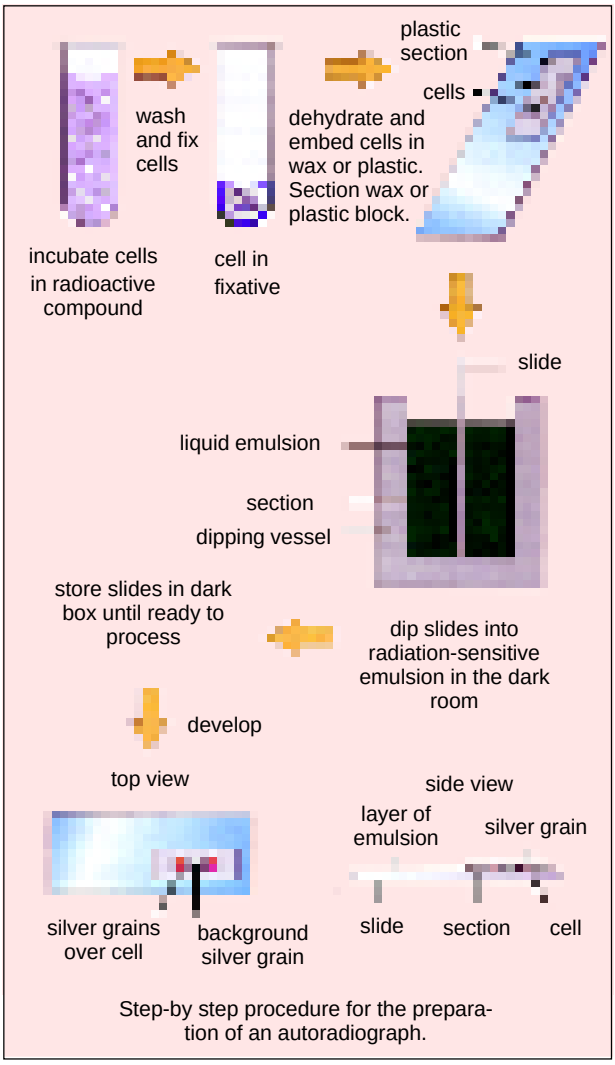
AUTORADIOGRAPHY

Autoradiography is a technique which is used to locate radioactive isotopes in cells, tissues, organs and whole organisms. A specimen is exposed to a solution containing molecules that have been made radioactive by the incorporation of radioactive isotopes, such as tritium (^{3}H), carbon 14 (^{14}C), phosphorus (^{32}P) and sulphur (^{35}S). The tagged molecules are often precursor molecules used by the cell in the synthesis of other needed molecules. At intervals, samples are removed from the solution; in case of smaller tissues, the samples are sectioned and mounted on glass slides or grids. The sections are then coated with a photographic emulsion and stored in the dark for periods ranging up to several months.

When a radioactive atom emits a beta particle (*i.e.*, electron) the photographic emulsion is affected in a manner similar to the exposure of a photographic emulsion to light. Over a period of time sufficient radioactive emissions occur to affect the silver grains of the emulsion. Black spots will appear at those sites when the emulsion is developed. Such spots will mark sites in the tissues where the radioactive atoms have accumulated. These sites can be identified by examining the stained tissue sections under the light microscope.

In the technique of autoradiography, for the study of DNA metabolism of cell ^{3}H-thymidine is used; for RNA metabolism ^{3}H-uridine is used; for protein synthesis various tritiated (^{3}H-tagged) amino acids are used; and for polysaccharides and glycoproteins tritiated monosaccharides such as ^{3}H- mannose and ^{3}H-fucose are employed.

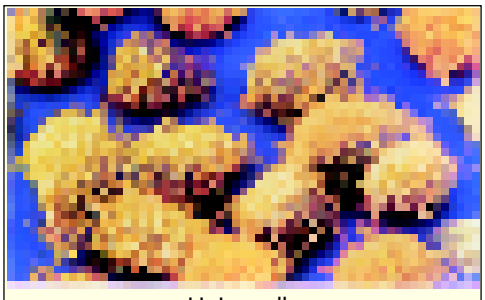
Pulse-labelling technique. This technique is used for those cases where biological molecules undergo considerable modifications after their synthesis (*e.g.*, ribosomal RNA). Here, actively growing cells are exposed to a radioactive precursor for a short period. The labelled precursor is then removed and replaced by 'cold' (unlabelled) precursor molecules. The unlabelled precursors are incorporated into the newly synthesized molecules and have the effect of 'chasing' the previously synthesized molecules containing the radioactive precursor through any maturation process. If the molecular species under investigation is sampled shortly after the start of the experiment, only the primary synthetic product will contain radioactivity. After longer time intervals, the original radioactive molecules will have been replaced with non-radioactive molecules. In this way the flow of radioactivity through a maturation process can be followed, together with any movement of molecular species within the cell.



Step-by step procedure for the preparation of an autoradiograph.

CELL CULTURE

For cell biological observations sometimes it is needed to keep the animal and plant cell in living state outside the organism under favourable conditions. This process is called **cell culturing**. The cell cultures are of three main types: primary, secondary and those using established cell lines. **Primary cultures** are those obtained directly from animal tissue. The organ is aseptically removed, cut into small fragments and



HeLa cells.

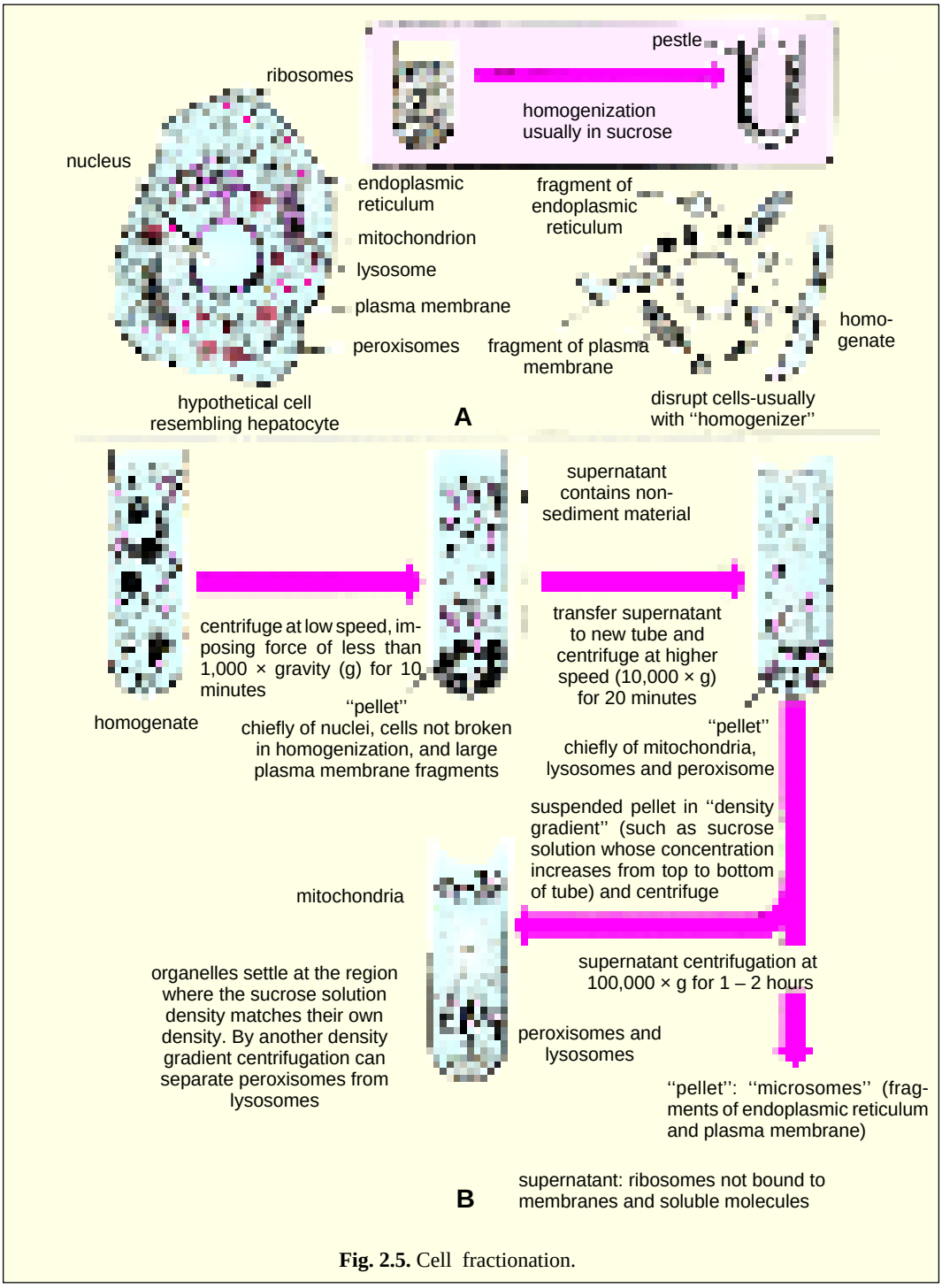


Fig. 2.5. Cell fractionation.

treated with trypsin enzyme to dissociate the cell aggregates into a suspension of viable single cells. These cells are plated in sterile petri-dishes and grown in the appropriate culture medium. When this culture is trypsinized and re-plated in a fresh medium then resultant culture is called **secondary culture**.

The other major type of cell culture uses **established cell lines**, which have been adapted to prolonged growth *in vitro*. Among the best known cell lines are **HeLa cells** (obtained from a human carcinoma), the **L** and **$3T_3$ cells** (from mouse embryo), the **BHK cells** (from baby hamster kidney) and the **CHO cells** (from Chinese hamster ovary).

CHROMATOGRAPHY

The chromatography is used to separate the molecules of different substances present together in a solution or cytosol. The solution is applied to an insoluble medium which has a different affinity for the individual molecules of the solution so that the molecules migrate through the medium at different rates. Following two types of chromatography are used in molecular biology :

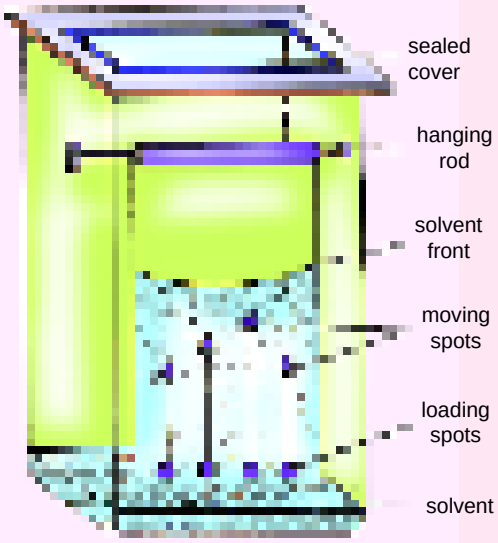
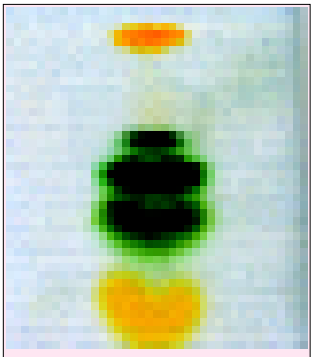


Fig. 2.6. Paper chromatography.

(A) Paper chromatography. The paper chromatography (Fig. 2.6) is a smiple method for the separation of smaller molecules from one another. The molecules to be separated are applied to sheets of suitable paper, which are subsequently placed in a vessel which contains a suitable solvent. A distinction is made between ascending and descending paper chromatography according to whether the solvent migrates on the paper from below or above. Highly soluble components of the sample mixture will migrate at the solvent front; other substances move more slowly according to their solubility. From this it follows that the choice of solvent determines the speed of migration of the individual molecules and is crucial for a successful separation. The paper chromatography method is used for the separation of amino acids, nucleotides and other lower molecular-weight metabolic products.



Thin layer chromatograph.

(B) Column chromatography. In column chromatography (Fig. 2.7), an insoluble medium is packed into a glass tube; the length and width of this tube influence the separation of the molecules. The molecules to be separated are applied to the top of the column and their migration is started by adding a solvent. The characteristic separation which results depends on the choice of solvent and carrier material. A positively charged carrier binds negatively charged molecules; other carriers contain pores which are penetrated by the smaller molecules, which are, therefore, slowed down. The solution which flows from the column is collected in small fractions, which contain the separated classes of molecules. Column chromatography is important for the separations of mixtures of proteins, that is, for the isolation of enzymes such as cytochrome C or RNA polymerase.

In a nutshell, presently the following types of chromatography are used for the isolation of different types of molecules (**King**, 1986; **Sheeler** and **Bianchi**, 1987):

A. Paper chromatography (used for separation of amino acids, nucleotides, and other low molecular weight solutes).

B. Thin layer chromatography or TLC (used for rapid separation of unsaturated and saturated fatty acids, triglycerides, phospholipids, steroids, peptides, nucleotides, etc.).

C. Column chromatography. It includes the following four types :

1. **Ion-exchange chromatography** (used for separation of proteins, RNA and DNA).
2. **Affinity chromatography** (used for separation of immunoglobulins, cellular enzymes, mRNAs).
3. **Gel permeation chromatography or Gel filtration** (Used for separation of proteins, nucleic acids, polysaccharides and lipids).
4. **Gas chromatography** (for the separation of lipids, oligosaccharides and amino acids).

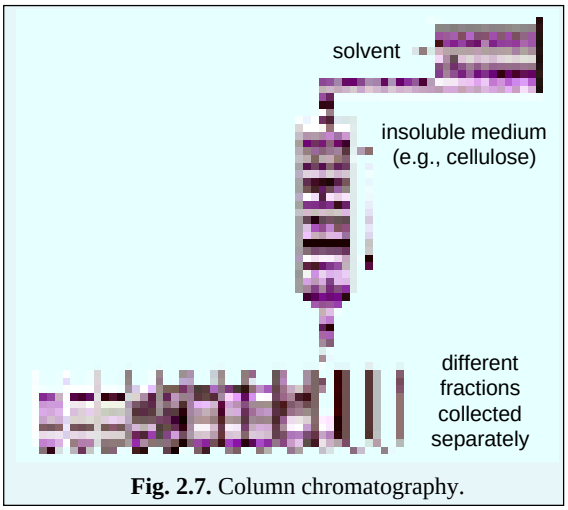


Fig. 2.7. Column chromatography.

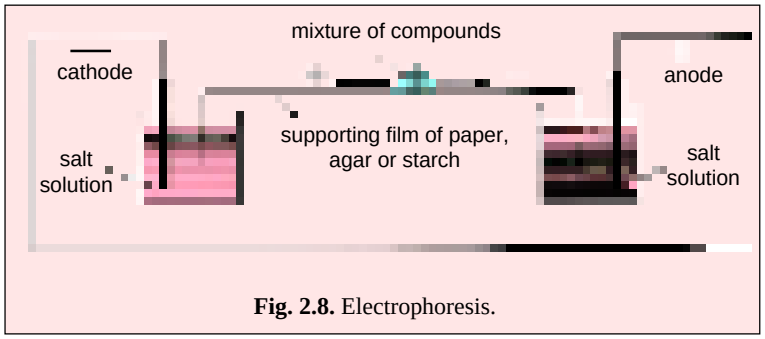
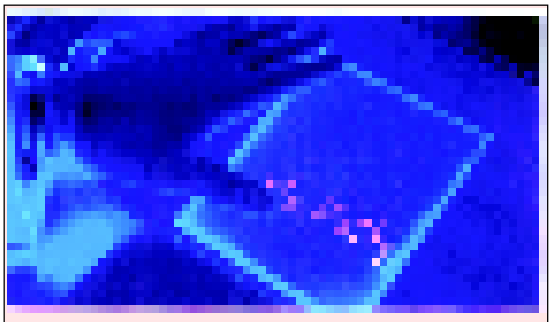


Fig. 2.8. Electrophoresis.

ELECTROPHORESIS

Molecules or macromolecules may be separated in an electric-field if they are charged to different extents. The mixture of compound is applied to supporting films which dip into two containers filled with a salt solution. One of the containers holds a cathode, the other an anode. On passing an electric current, the negatively charged molecules migrate to the anode and the positively charged molecules to the cathode. Paper, agar and starch are examples of substances which may be used as supporting films.

The rate of migration of a molecule in an electric field is determined by its size and the number of charged groups per molecule. The electrophoresis method is used in the separation of proteins, nucleic acids and their building blocks.



Separation of DNA restriction fragments by gel electrophoresis.

Some of the common types of techniques of electrophoresis, which are currently used in cell biology, are the following (**Sheeler** and **Bianchi**, 1987; **Alberts** *et al.*, 1989):

1. **Moving-boundary electrophoresis** (used for proteins).
2. **Gel or zone electrophoresis** (used for proteins).
3. **Discontinuous electrophoresis** (used for isolation of proteins of plasma membrane).
4. **SDS-PAGA** or **Sodium dodecyl sulphate-polyacrylamide gel electrophoresis** (used for separating and sizing macromolecules such as proteins, *e.g.*, membrane proteins, protein component of cytoskeleton, etc.).

5. Maxam - Gilbert technique (used for separation of polynuclotide fragments of RNA and DNA).

6. Immunoelectrophoresis (used for antigens and antibodies).

DIALYSIS

It is a sensitive method for separating lower molecular-weight component from macromolecules. A thin membrane in the form of a tube is filled with the solution containing the molecules to be separated. The pore size of the membrane allows the diffusion of small molecules such as salt or amino acids; larger molecules such as proteins or nucleic acids cannot pass through the pores and so remain inside the dialysis tube.

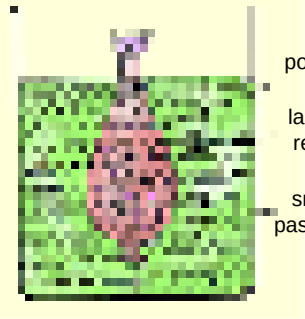


Fig. 2.9. Dialysis.

REVISION QUESTIONS

1. Enumerate various types of light microscopy. Describe the phase contrast microscopy.
2. Give a comparative account of light microscopy and electron microscopy.
3. Define the following terms : resolution, magnification, birefringent, vital stain, electron stain, cell fractionation, chromatography and electrophoresis.
4. What ways are available to improve contrast in light microscopy ?
5. Describe different techniques used in preparing sample for electron microscopy.
6. Write short notes on the following :

(i)	Cytochemical stains;	(v)	Autoradiography;
(ii)	Scanning electron microscope;	(vi)	Cell culture;
(iii)	X-ray diffraction analysis;	(vii)	Chromatography; and
(iv)	Cell fractionation;	(viii)	Electrophoresis.