TECHNIQUES IN CELL BIOLOGY

Advancements in Cell Biology are due to introduction of new tools and techniques in the study of cell. Few are them are discussed below.

1) Microscopy: The diameter of most cells fall in the size range of 0.2 μto 50μm, which is much below the resolving power of human eye. Limit of resolution (l) is defined as the smallest distance that may separate two points on an object and still permit their observation as distinct separate points. Magnification of images/objects increases resolution limits and human eye cannot see objects such low diameters. A light compound microscope having many lenses can magnify objects upto 1500 times. The limit of resolution (l) of an optical instrument is given by Abbe's relationships:

$$\text{Resolution (l)} = \frac{\text{Wave length } (\lambda)}{\text{Numerical aperture } (n \sin \alpha)}$$

Where, n = refraction index of material

Sin α = Sine of the angle of aperture of the first lense

Refractive index is increased by using immersion oil which are transparent. A good light microscope, with a numerical aperture of 1.4 and using light of short wave length (0.4 μm) will resolve two points at ~0.17 μm separation.

Light Microscopy:

Compound Light Microscope uses visible light for illuminating objects. They contain glass lenses that magnify objects and focus light on the retina of eye of observer. The lense closer to eye is called the Eye Piece or Ocular lense and the lense closer to object is called the Objective lense (figure 2.1). A condenser lense between light source and object serves to focus light on the object.

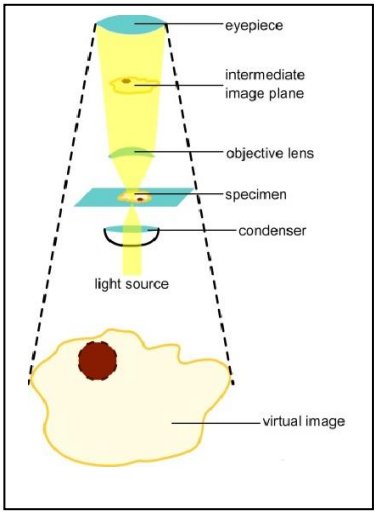


Figure 2.1: Principle of Light Microscope

Improved Types of Light Microscopes:

1) Dark Field Microscope or Ultra microscope: It is useful in viewing suspensions of bacteria. In this Microscopy, the object is viewed using oblique rays and light rays scattered by objects are viewed. Hence the image appears bright in dark background (Figure 2.2).

2) Phase Contrast Microscope: Different parts of the cell have different densities and hence different refractive indices. In the regions where refractive index changes, the light tends to bend. These bent rays are used to from patterns of destructive interference, yielding sharp contrast images. This microscopy is widely used to observe actively dividing cells which are stained.

Interference Microscope is based on the principle of Phase contrast microscopy and it permits detection of small and continuous changes in refractive cells.

3) Polarization Microscope: This is used for viewing objects such as crystals or bundles of parallel filaments etc., using polarized light. If the material is isotropic, the polarized light is propagated through it with the same velocity, independent of the impinging direction. These substances are characterized by having the same index of refraction in all directions. In the case of Anisotropic materials, the velocity of propagation of polarized light varies. These are called Birefringent materials as they present two different indices of refraction corresponding to the respective different velocities of transmission. In this microscopy, the specimen is placed between two closed polarizers and visible birefringent portions of the sample acct life polarizing films, and hence, these portions of the sample are seen as bright objects on dark back ground.

Figure 2.2: Image seen using Ultramicroscope

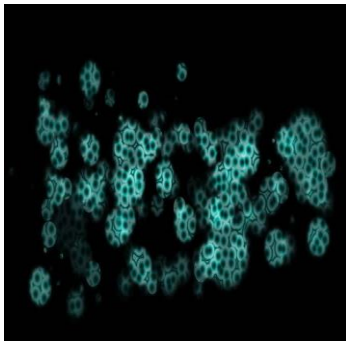
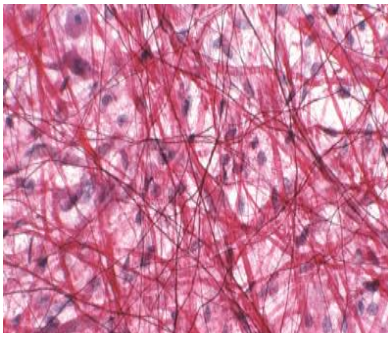


Figure 2.3: Image seen using Phase Contrast Microscope

Figure 2.3: Image seen using Polarization Microscope



Sample preparation for Light microscopy:

The steps involved in sample Preparation for light microscopy includes: Killing cells, Fixation, Dehydration, Embedding, Sectioning, Staining and mounting of slides. Stains used in these studies are of significance as they make the parts stand out among others. These are of two types, acid stains- which include Eosin, Orange G, Aniline Blue and Fast green. Basic stains include- Methylene Blue, Crystal violet, Haematoxylin, Basic Fuchsin etc. In addition, there are certain specific stains called as Cytochemical stains, that selectively bind to specific groups of macromolecules in a cell. These include- Millon reaction, Diazonium reaction and Naphthol Yellow for proteins; Alkaline fast green for Histone proteins, Feulgen reaction for DNA, Methylene Green- pyronine stain to distinguish DNA and RNA (DNA stains green and RNA stains red)(Figure 2.5), Acetocarmine to stain chromosomes in dividing cells (Figure 2.6), Sudan Black and Sudan Blue for lipids etc.

Figure 2.5: Staining of cells by Methylene Green-Pyronine showing DNA in green and RNA in red

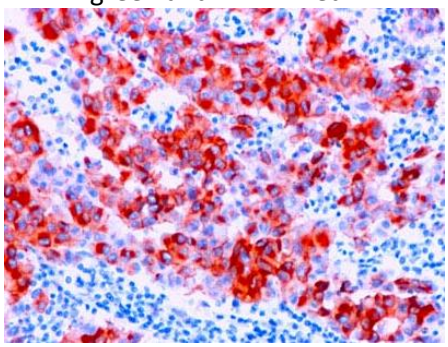
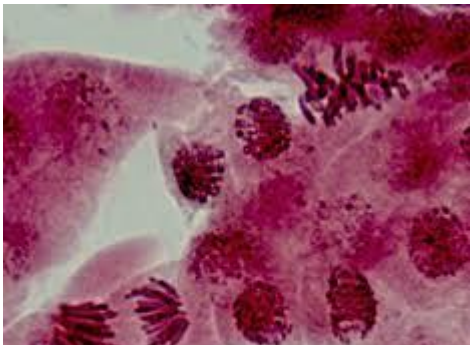


Figure 2.6: Chromosomes stained by acetocarmine



Electron Microscopy:

The Electron microscopy uses short wave lengths of electron to view sample and achieve a resolution as low as $3A^{o}$ (Figure 2.7). This technique uses Electromagnetic coils that are used to control and focus a beam of electrons accelerated from a heated metal wire by supplying high voltage in the range of 20,000 to 1,00,000 volts. The wave length of electrons depends on the magnitude of voltage applied and the wave length is around $0.01A^{o}$. These electrons are scattered by specimen, which is placed in the path of beam. Various imaging principles are used to generate final image of the specimen the sample scatters the electrons and this scattering depends on density of regions within it. Therefore, regions which are of less density appear as dark regions and those of high density as lighter regions.

Specimen preparation is important in this transmission electron microscopy and they are Ultra thin sections of 10nm to 100nm thick (Figure 2.8). The samples are generally viewed directly, but to increase contrast, electron stains such as Urynyl acetate, Uranyl citrate, Lead citrate, Osmium tetroxide etc are used. To view small particles life viruses, negative staining is

done using Phospho tungstic acid that penetrates into all empty spaces of the specimen and increases the contrast.

Scanning transmission Electron Microscopy (STEM):

It has less resolution power than TEM (~200A^{o}) and is an effective tool used to study surface topography of specimens. A narrow beam of light is scanned rapidly over specimen, and it results in 3d image from the differential scattering of electron by different part of specimen.

Figure 2.7: Comparison of Light Microscope, Transmission Electron microscope and Scanning Transmission Electron Microscope

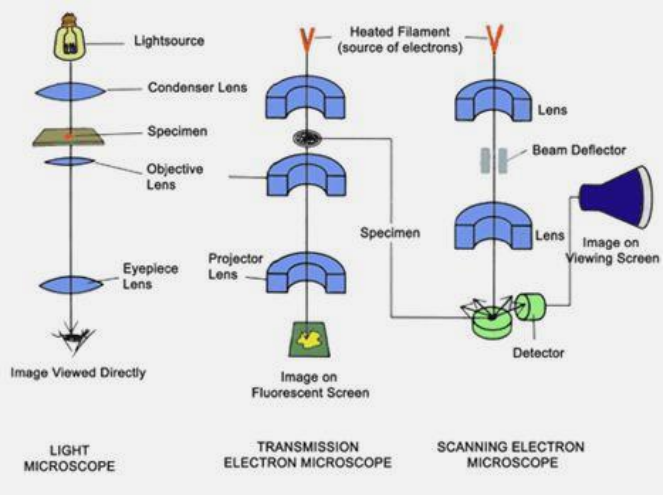
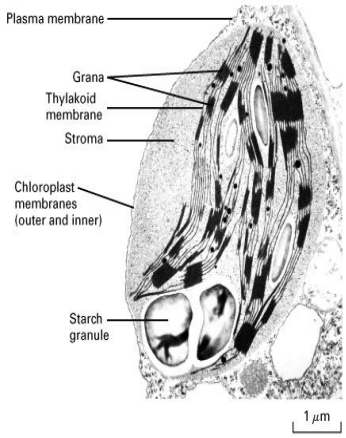


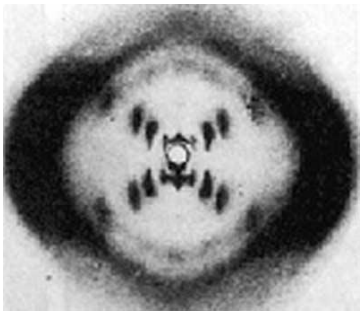
Figure 2.8: Specimen viewed by TEM



X-ray Diffraction Analysis:

This technique is used to analyze 3d structure of DNA molecule and many proteins such as myoglobin, Haemoglobin, Collagen, Myelin sheath of nerve cell etc. This method depends on principle of scattering or diffraction of X-rays by atoms of substance.
If the material has an ordered crystalline atomic structure, the resulting X-ray diffraction pattern is also ordered and reflects the 3d arrangements of atoms in crystals (Figure 2.9).

Figure 2.9: X-ray diffraction pattern of DNA

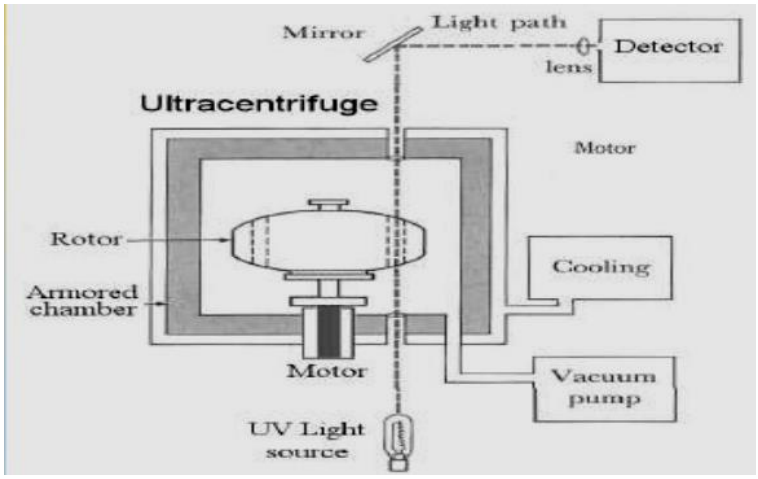


Cell Fractionation:

These techniques are used to study subcellular components, study tissues, cells etc. The procedure involves homogenization, destruction of cell boundaries, followed by sub cellular fractionation based on mass, surface and specific gravity by centrifuges. The centrifuges used in Cell Biology are **Analytical Ultracentrifuge** that provides information pertaining to mass and shape of molecules(Figure 2.10), while the other is **Preparative Ultracentrifuge** that can be used for separating molecules.

An ultracentrifuge with rotor of high rpm of around 70,000 revolutions per minute is used. Analytical ultracentrifuges contain optical system, allowing one to observe changes in solute of the solution. As they spin at high speed, the rotors are placed in vacuum to prevent generation of heat.

Figure2.10: Schematic representation of Analytical Centrifuge



The Procedure involves cell fractionation, tissue disruption or cell disruption and homogenization. The homogenate is then subjected to differential centrifugation in sucrose solution with increasing velocities (Figure 2.11). The principle in differential centrifugation is that at each step, particles settle down based on their rates of sedimentation. After all the organelleles settle down, the residual solution is called **Cytosol** which contains the soluble macromolecules of cells. These can be isolated using biochemical techniques like chromatography, dialysis and electrophoresis.

Density Gradient Centrifugation is best suitable for all fractionation. In this method, anincreasing gradient of solution is layered in centrifuge tube using Cesium Chloride, Sucrose, Heavy water or albumin. As the homogenate is layered on top and subjected to centrifugation, particles tend to migrate to various distances along the tube based on their buoyant densities (Figure 2.12). This type of separation is called Equilibrium density or Isopycnic centrifugation.

Figure 2.11: Step by step procedure of cell fractionation by Differential Centrifugation

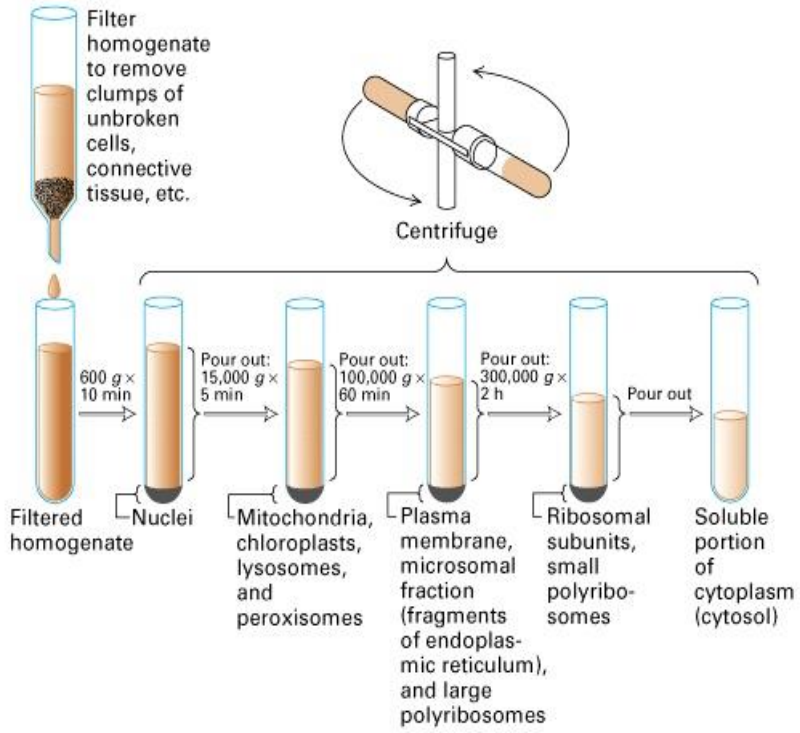
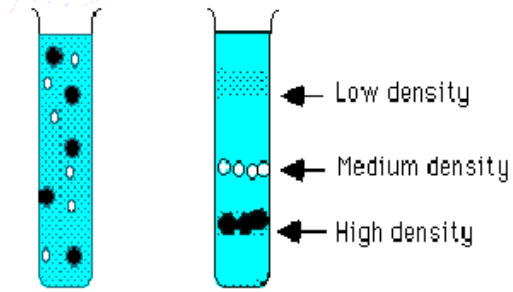


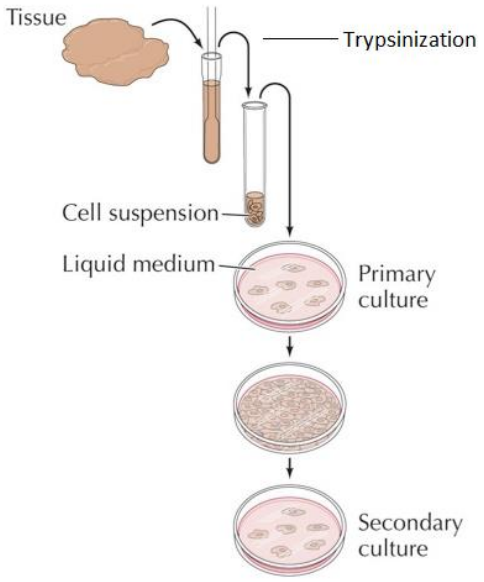
Figure 2.12: Isopycnic centrifugation of protein that get separated based on their buoyant densities.



Cell Culture:

Biological studies required growing cells outside their natural existence, like plants and animal cells and this is called Cell Culturing. It involves providing favorable conditions invitro for proper growth of cells. Cell cultures are of three types: Primary cell cultures, Secondary cell cultures and established cell lines. Primary cell cultures are obtained from plant or animal tissues or organs. A free cell suspension is prepared by using appropriate disaggregation methods, for example in the case of animal cell culture; the tissue is subjected to trypsinization. These are grown in Petri plates and then subjected to another cycle of trypsinization and plating in fresh medium. The resultant culture is called Secondary culture (figure 2.13). Prolonged growth of cells in culture will result in cell lines, examples of which include He La cells (Obtained from Human carcinoma), the L & $3T_3$ cells (obtained from mouse embryo) and the BHK cells (from Baby Hamster Kidney).

Figure2.13: Formation of Primary and Secondary cell cultures from animal tissue



Chromatography:

These techniques are used to separate mixture of molecules into their component. The following are the commonly used chromatography techniques in Cell Biology.

A) Paper Chromatography: It is a planar chromatography that uses the principle of partition coefficient. In this technique high quality Whatman filter paper is used to separate samples Figure 2.14). Based on the solubility of sample between stationary phase and mobile phase the same migrates to varied distances on the paper. Water which adheres to the cellulose and act as stationary phase while mobile phase is any solvent that offers solubility to solutes under study. There are several types of paper Chromatography, like the Ascending chromatography, Descending chromatography and radial chromatography. 2-dimensional chromatography enables to separation of complex mixture of samples and is conducted in two dimensions using two different samples. This procedure involves determination of Rf values that are constant for a sample under standard conditions and helps to identify unknown solutes in the test.

Thin layer chromatography is another planar chromatography which used thin layer of Activated alumina or silica gel-G as stationary phase (figure 2.15). These materials are coated on to a plane surface of glass or aluminum sheet and samples are applied. A closed chamber having solvent system is taken and the plates are placed in it. As the mobile phase ascends the samples migrate based on adsorption. Paper chromatography is used for separation of amino acids, Nucleotides, pigments carbohydrates and low molecular weight solutes, while TLC can be used for rapid separation of saturated and unsaturated fatty acids, triglycerides, phospholipids, steroids, peptides, nucleotides etc.

Figure 2.14: 2-D paper chromatography peocedure

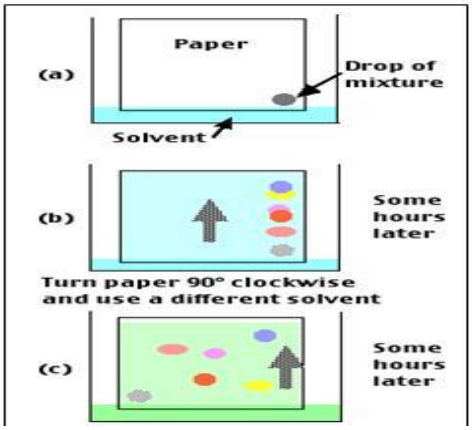
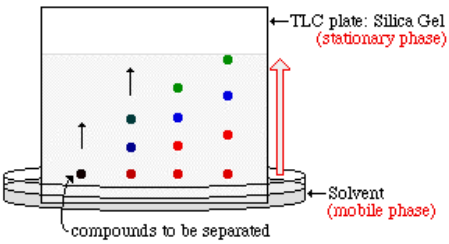
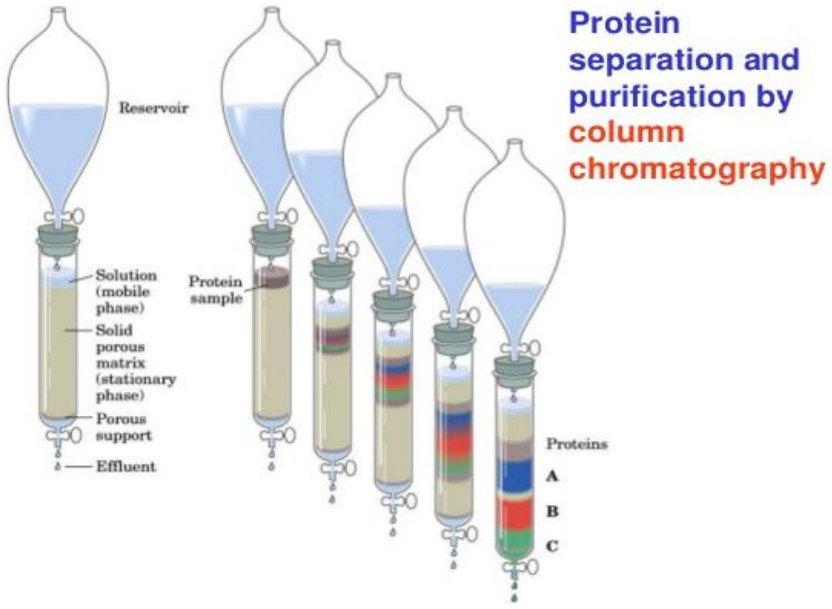


Figure 2.15: Thin layer chromatography



B) Column Chromatography: In these set of chromatographic techniques columns packed with various materials are used for separation of mixtures. The general components of column chromatographies include- column packed with matrix (which varies based on principle), solvent reservoir, suitable solvent, fraction collection system followed by analyzer (Figure 2.16). These enable qualitative analysis of mixtures as well as for preparative purposes. Based on requirement the column dimensions vary. In case of GC they are long and narrow, while in gel permeation and Ion exchange chromatographies it is much larger.

Figure 2.16: Column chromatography illustrating separation of protein mixture



The most used column chromatographies include:

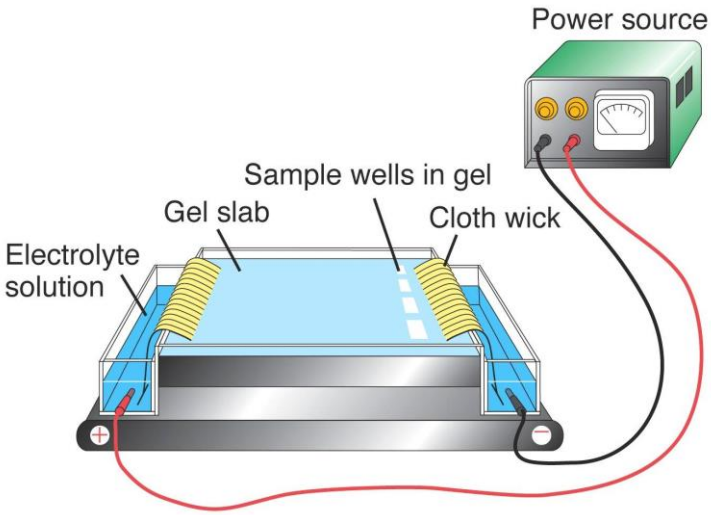
1) Ion exchange chromatography: Used for the separation of charged molecules like Proteins, RNA and DNA.
2) Affinity chromatography: Used to separate molecules having specific binding like, Immunoglobulins, Enzymes, mRNA's etc.

3) Gel permeation chromatography: Separates molecules based on size, and is used to separate proteins, Nucleic acid.
4) Gas chromatography: Uses gas as a mobile phase and is used to separate lipids, oligosaccharides and aminoacids.

Electrophoresis:

It is one of the important tools in cell biology which is rapid, inexpensive that is used to separate charged molecules. This method used support medium which is a porous gel. As molecules like DNA, RNA or proteins move through the gel, based on charge or charge to mass ration mixture gets separated (Figure 2.17). The separated samples are visualized using appropriate methods of visualization. This method has several variations which include;

Figure 2.17: Outline view of components of Gel Electrophoresis.



1) Moving Boundary Electrophoresis: Separation of samples takes place in solvent system, and is used for separation of proteins.
2) Gel or Zone electrophoresis- used for separation of proteins and nucleic acids
3) Discontinuous Electrophoresis- for isolation of proteins from plasma membrane
4) SDS-PAGE- Separation of proteins based on size, as charge is masked by SDS (sodium dodecyl sulphate- polyacrylamide gel electrophoresis)

5) Maxam-Gilbert technique- used for separation of polynucleotide fragments during their sequence determination.
6) Immuno electrophoresis: Used for separation of antigens and antibodies.

Dialysis:

It is the process of separating molecules in solution based on difference in their rate of diffusion across a semipermeable membrane (Figure 2.18). This laboratory technique employs usage of dialysis bag having a pore size which determines the diffusivity of molecules. The molecules whose diameter is less that the pore size of the membrane will diffuse out of the bag. It is used in protein purification, removal of salts from precipitates, removal of unwanted small ions, salts, sugars, reducing agents or dyes from larger molecules like proteins, polysaccharides or DNA.

Figure 2.18: Principle of dialysis