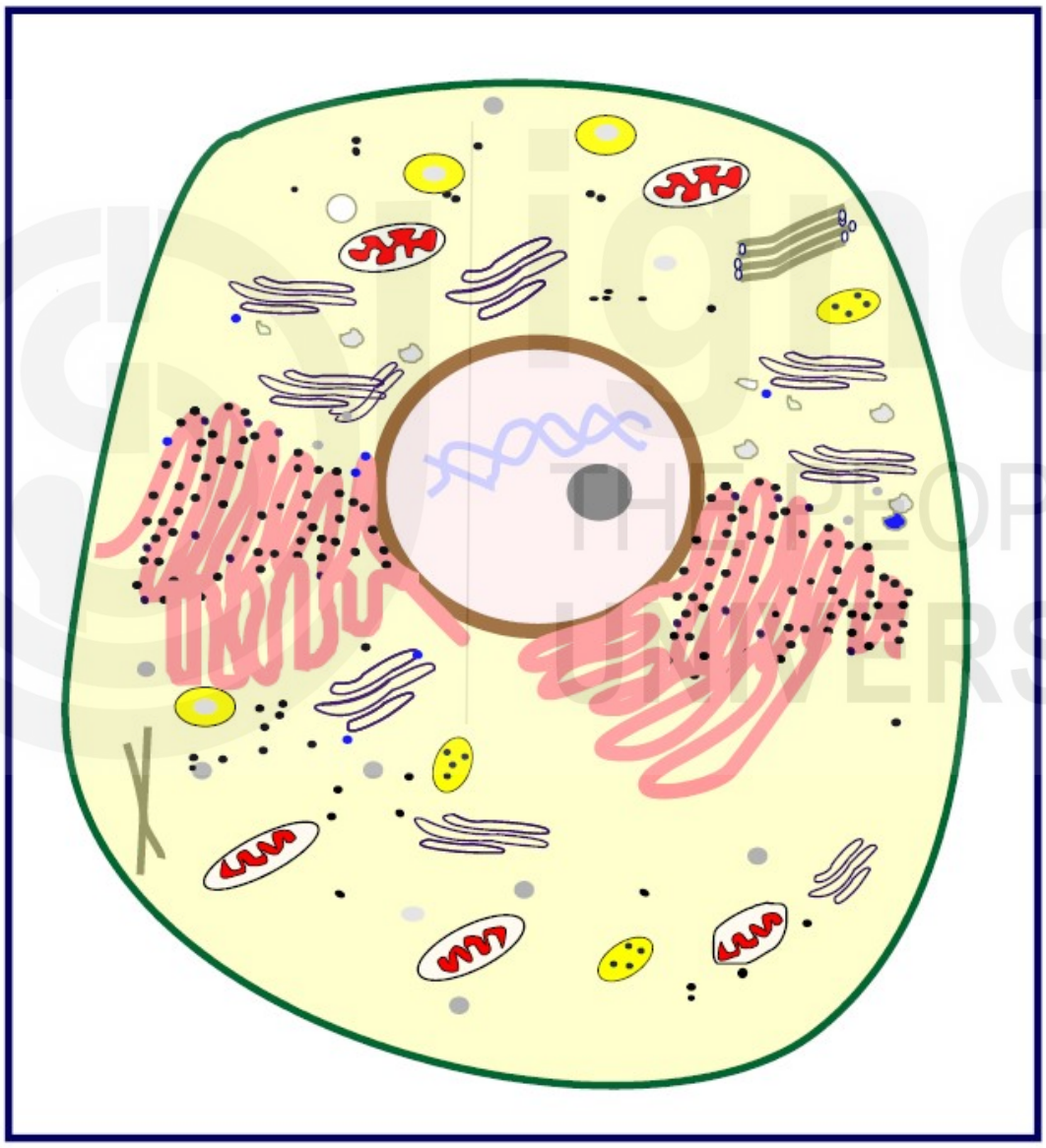
BBCCT - 103

CELL BIOLOGY

Indira Gandhi National Open University
School of Sciences



Indira Gandhi National
Open University
School of Sciences

BBCCT-103
CELL BIOLOGY

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CELL BIOLOGY: COURSE INTRODUCTION

Cell Biology is the Core course II of the B.Sc. (Hons.) Biochemistry Programme (BSCBCH) being offered under UGC-CBCS Scheme. The theory course of Cell Biology (BBCCT-103) has 4 credit weightage and the laboratory course (BBCCL-104) is of 2 credits. While preparing this course,we have kept in mind that students joining our B.Sc. Honours Biochemistry Programme must have studied subjects i.e. Biology, Chemistry, Physics upto 10+2 level.As progress in the modern science, Cell Biology has emerged and developed as a core subject of Biochemistry with simultaneous advances in the Microscopy field which made remarkable discovery and contribution to understanding the chemistry of the cell and much more remains to be discovered.
This theory course consists of 4 blocks which contain 13 Units.

Block 1: Introduction to Cell Biology discusses the cell theory, structure and basic features of the prokaryotic (Archaea and Bacteria) and the eukaryotic cells (Animal and Plants) along with the cell as an experimental model. You would be introduced to basic techniques and application of the Optical Microscopy, Electron Microscopy and Centrifugation that are used to study Cells.

Block 2, describes the structure and functions of subcellular organelles: Nucleus, Endoplasmic reticulum, Golgi Body, Peroxisomes, Lysosomes, Mitochondria and Chloroplast. It is also described the Cell wall and Extracellular metrix, Cytoskeleton proteins and Cell movement.

Block 3: Protein Transport focuses the targeting of proteins to the subcellular organelles in which you will learn how a secretory protein transports and incorporates into the ER membrane or across the ER membrane. This block gives you brief introduction to the protein transport and explains how signal sequences of different proteins target and transport from their site of synthesis to their action site in a cell *i.e*. the nucleus, the mitochondria etc.

Block 4: Cell cycle and its regulation discuss the different phases of cell division: Mitosis and Meiosis. This block also explains the Cell death and cell renewal.

This course is being offered at the first semester and will build up foundation for the other courses which you will study in the other semesters.

Each unit in the course contains expected learning outcomes, text summary and self assessment questions. You are advised to attempt all the self assessment and terminal questions given in this block as they help in understanding important concepts in the unit. You are also advised for attending counseling sessions, watching the audio-video programme and participating in IRC/teleconferencing session related to this course.

Objectives

After studying this course, you should be able to:

- define the cell and cell theory
- classify the cell;
- describe structural organization of prokaryotes and eukaryotes and differences between them;
- explain the principle and application of light and electron microscopy and centrifigation;
- describe the structural and functions of sub-cellular organelles;
- explain the structural organisation of cell wall, extracellular matrix and cell-cell interactions;
- explain the structural component cytoskeleton proteins and their role;
- describe pathways of protein transport and signal hypothesis;
- define the meiosis and mitosis;
- explain the different phases of mitosis and meiosis of the cell division and its regulation;
- illustrate the cell death and cell renewal; and
- describe the principle and application of fluorescence activated cell sorting (FACS) technique

We hope you will find this course quite interesting to study.

Our best wishes:

Indira Gandhi
National Open University
School of Sciences

BBCCT-103
CELL BIOLOGY

Block

1

INTRODUCTION TO CELL BIOLOGY

UNIT 1
The Cell 07-28

UNIT 2
Optical Microscopy 29-48

UNIT 3
Electron Microscopy 49-64

UNIT 4
Centrifugation 65-86

BLOCK 1 : INTRODUCTION TO CELL BIOLOGY

This block consists of 4 units dealing with the Cell, Optical microscopy, Electron microscopy and Centrifugation.

Unit 1 describes the cell and how cell theory evolved by the microscopic invention to an interdisciplinary subject. A detailed discussion on structure, organization and function of the prokaryotic cell and the eukaryotic cell has been dealt.

Unit 2 deals with the Optical microscopy. It describes the principle of microscopy and the basic set up of a compound microscope. This unit explains the methods of cell fixation and types of Light microscopy- Bright and Dark Field microscopy, interference and phase contrast microscopy, fluorescence microscopy, confocal microscopy and their applications.

Unit 3 deals with the working principle, Instrumentation, theory and application of Transmission Electron Microscope (TEM) and Scanning Electron Microscope (SEM). This unit also describes the preparation of biological specimen to be examined under the Electron microscopy.

The Unit 4 talks about the basic principle, instrumentation and application of centrifugation. It also describes the different types of centrifuge and centrifugation techniques like differential centrifugation and density gradient centrifugation.

Objectives

After studying this block, you should be able to:

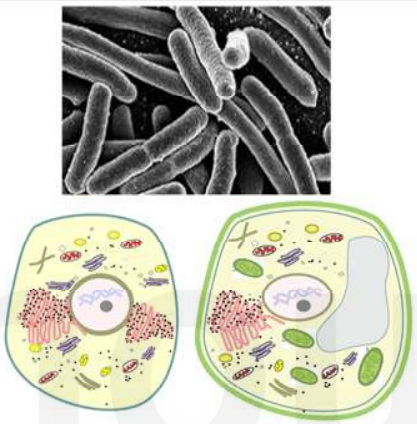
- describe the cell, cell theory, structure of the prokaryotic and the eukaryotic cells;
- distinguish special differences and general features between animal and plant cells;
- list the general features of the cell as an experimental model;
- understand the basic principles of microscopy (optical microscopy and electron microscopy);
- list the basic steps for the preparation of biological specimen;
- explain the principle, instrumentation, types of centrifugation; and
- describe the differential and density gradient centrifugation and their application.

UNIT 1

THE CELL

Structure

1.1 Introduction
Expected Learning Outcomes
1.2 Cell theory
Classification of the Cell
1.3 Prokaryotic Cell
Archaea
Eubacteria
1.4 Eukaryotic cell
Animal and Plant Cells
Comparison of the Three Major Groups (domains) of Organisms.
1.5 Cells as Experimental Models
1.6 Summary
1.7 Terminal Questions
1.8 Answers
1.9 Suggested Readings



1.1 INTRODUCTION

This is the first Unit of Block 1 entitled "**Introduction to Cell Biology"**. Cell biology is the branch of science that deals with the study of cell structure and function. The understanding of life processes and how cells respond to the changing needs is an essential aspect of cell biology. The molecules of life that make up cells include proteins, carbohydrates, lipids, nucleic acids (DNA and RNA) and vitamins about which you have studied in the Course BBCCT-101 "Molecules of Life". In addition all biological processes and interactions take place in aqueous environment.

When we look around us we come across a variety of living organisms such as plants and animals. Have you ever thought what makes them living? The answer is the cell. A cell is the smallest fundamental unit of life that is capable to performing all life processes. Most cells are microscopic in size and all living organisms are composed of either one (unicellular) or more (multicellular) cells. They are further subdivided into prokaryotes or eukaryotes depending on whether they possess or lack a true nucleus and membrane bound organelles respectively.

Therefore, in this unit you shall learn about the cell. The unit begins with the cell theory and classification of the cell. Thereafter you shall learn about the structural organization of the prokaryotic cell (archaea and eubacteria) and the eukaryotic cell (animal and plant cells). The characteristic differences between archaea, bacteria and eukaryotic cell are also summarised. At the end of the unit, the cell and organisms as an experimental model are described.

The next three units of Bock-I will deal with Optical Microscopy (Unit 2), Electron Microscopy (Unit 3) and Centrifugation (Unit 4).

Expected Learning Outcomes

After studying this unit, you should be able to:

- define the cell and the salient features of the cell theory;
- discuss the structural organization of prokaryotic cells;
- explain the structural organization of eukaryotic cells;
- compare (a) prokaryotic and eukaryotic cells and (b) animal and plant cells;
- identify the cells based on their characteristic features; and
- indicate the suitability of different cells/ organisms as an experimental model.

1.2 CELL THEORY

This section focuses on the cell theory and classification of the cell. A Cell is the basic unit of life. This simple statement has emerged from the contribution of many investigators spanning more than two centuries. A cell can grow, reproduce, perform essential metabolic functions, responds to signals and adapts to changing needs.

Cells are not visible to the naked eye. They were first observed in the late seventeenth century with crude microscopes. With the improvement in the quality of microscope lenses it was possible to visualise cell structure and processes in much greater details. We will now consider some of the key discoveries that led to the formulation of the cell theory.

In 1665, the English natural philosopher **Robert Hooke** viewed thin slices of cork under the microscope and observed tiny compartment or space surrounded by walls that resembled a honeycomb. He called them cells from the Latin word 'cellula' meaning small compartment. What he actually saw was the dead cell walls of cork (Fig. 1.1).

Fig. 1.1: The small compartment (cells) observed by Robert Hooke.

Antonie van Leeuwenhoek was a Dutch businessman and a self taught biologist. He made optical lenses and some of his microscopes could magnify up to 500 times that made him to observe objects in greater details. He was the first scientist (in 1674) to see living cells in a drop of water and discovered animalcules (protozoa). He also observed bacteria, spermatozoa and red blood cells.

Robert Brown was a Scottish botanist. He is known for the discovery of the nucleus (1838) and Brownian motion.

Matthias Jakob Schleiden was a German Botanist who studied different plant tissues and concluded that they are all made up of cells (1838). He postulated that a cell is the basic unit.

Around the same time another German physiologist **Theodor Schwann (1839**) examined animal tissues and extended to animals the cell theory. Both of them did not explain how new cells are formed. The gap was filled in 1855 by a German doctor **Rudolf Virchow** who proposed that new cells arise by the division of pre-existing cells. This idea was originally given by Robert Remak.

The cell theory grew from the work and research of many scientists.

Schwann, Schleiden and Virchow are generally credited for the cell theory. The box below summarizes the salient features of the cell theory. It is a unifying concept of biology.

The Cell Theory

- **All living organisms are made up of one or more cells.**
- **A cell is the structural and functional unit of life.**
- **New cells arise by the division of pre existing cells.**

You might have studied about the Cell structure; function and its origin in your Biology Course at the 10+2 level. The earliest forms of life were single celled organisms that lived in an anaerobic environment. They arose almost 3.5 billion years ago on mother Earth. All other organisms evolved from these simplest forms and therefore are evolutionarily related to one another.

1.2.1 Classification of the Cell

Once the cell theory was an accepted idea in biology and with the continued improvements in the microscopes, the visualisation of sub cellular structures of cells became possible. It was soon recognised that all cells fall

into two broad categories depending on whether or not they contain a truenucleus and subcellular organelles. The term prokaryotic and eukaryotic cells were proposed by Hans Ris in the 1960s on the basis of their complexity. A given organism is made up of either prokaryotic or eukaryotic cells.

A **prokaryotic cell** such as bacteria lacks a true nucleus and **other membrane bound organelles**. The genetic material exists in the form of nucleoid in the cell cytoplasm whereas **eukaryotic cells** have a true nucleus and many membrane bound organelles (Fig. 1.2). All our present-day animal and plants are eukaryotes.

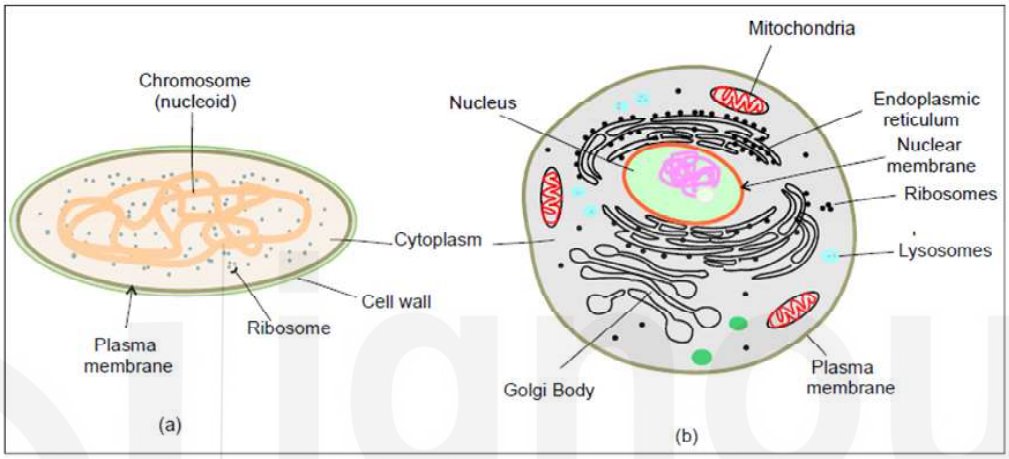


Fig. 1.2: (a) Prokaryotic cell, (b) Eukaryotic cell

Later studies led to the division of prokaryotes into two groups on the basis rRNA sequences. Therefore, all living organisms fall into one of the three large groups (domains): **Bacteria, Archaea** and **Eukaryota**. The phylogenetic tree in Fig. 1.3 shows the evolutionary relations among the three major domains. They all represent three branches of evolution from a common progenitor. The available evidence suggests that Archaea and bacteria evolved early in evolution and the third domain, Eukarya evolved from the same ranch that gave

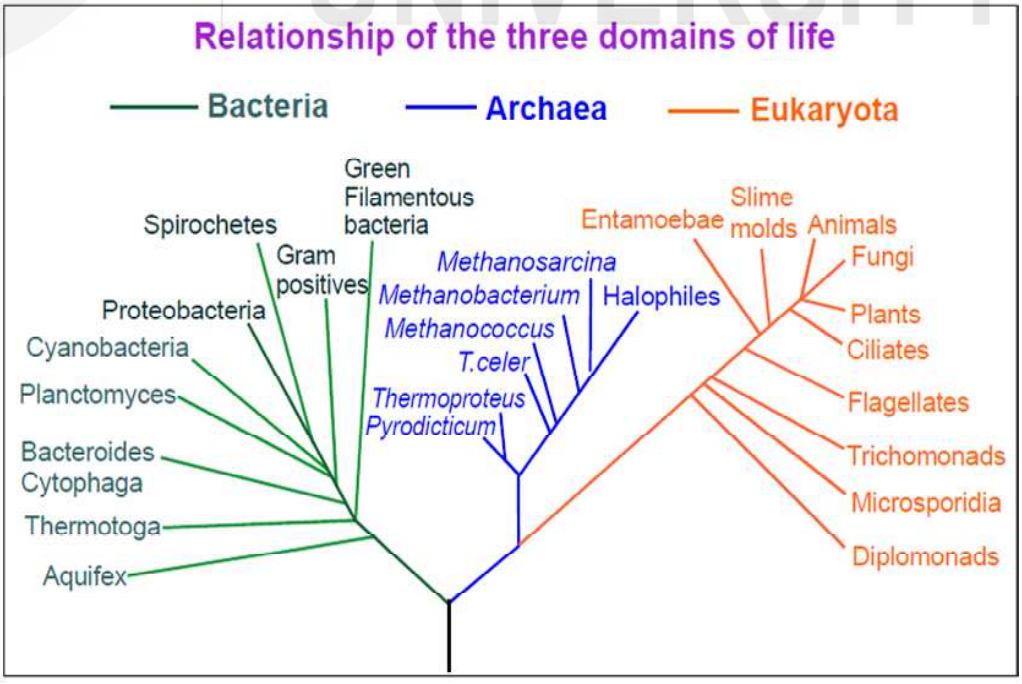


Fig. 1.3: Phylogenetic tree of life.

rise to Archaea. Eukaryotes are therefore more closely related to Archaea than to the bacteria.

You will study in details the structural diversity, distribution and specialised roles of all three groups in the following sections.

SAQ 1

i) Which one of the following statement is incorrect? Point out the error.

a) Robert Brown discovered the cell.

b) Schleiden and Schwann formulated the cell theory.

c) Virchow explained that cells are formed from pre-existing cells.

d) A unicellular organism carries out its life activities within a single cell.

ii) What is the basis of classification of cells?

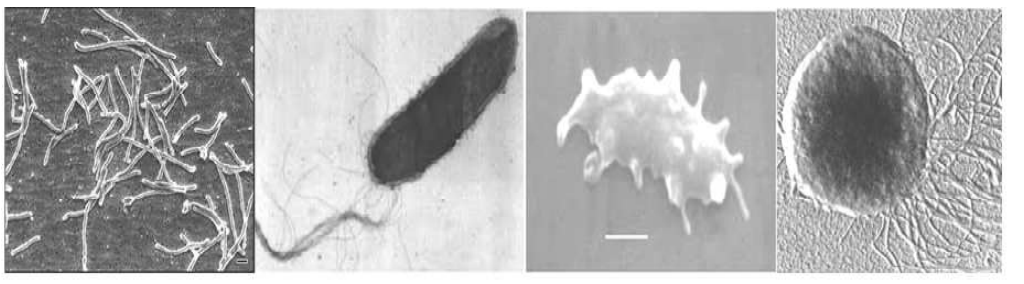
1.3 PROKARYOTIC CELL

The term Prokaryotic means before nucleus in Greek (Pro means **"before"** and karyon means **"nucleus")**. The prokaryotic cell typically lacks a true nucleus and membrane bound organelles. In this section you will study about prokaryotic organisms. As mentioned earlier prokaryotes are classified into two groups: **Archaea** and **Eubacteria.** Let us discuss one by one the characteristic features of both classes of prokaryotes.

1.3.1 Archaea or Archaeabacteria

The term "Archaea" is derived from the Greek word meaning "ancient ones". They are probably the first forms of life on earth dating back to approximately 3.5 billion years. Archaea may be single celled or form filaments. Like prokaryotes they do not have a **true nucleus** and membrane bound organelles. Their genetic material is DNA. They divide by binary fission, budding, fragmentation or other ways.

The group archaea is extremely diverse in their morphology, habitat, reproduction and physiology. They can be spherical, rod shaped, spiral, and irregular and even pleiomorphic (lack cell wall). Fig.1.4 shows the variations in their morphology.



Thermus aquaticus *Halobacterium salinarium* *Thermoplasma* *Methanococcus*

Fig. 1.4: Morphological variations in Archaeal organisms.

Many of them can survive under extreme conditions such as high temperatures, low pH, high salt, and high alkali or a combination of more than one of these physical conditions. Due to their ability to withstand extreme conditions they are called **extremophiles.** Table 1.1 highlights the categories of representative extremophiles along with the ecological niches they are generally abundant. Some of them are also part of the human microbiota. Based on their oxygen dependence; some are aerobic whereas others are either obligate anaerobes or facultative anaerobes.

Table 1.1: Classification of extremophiles

Extremophiles	Examples	Extreme conditions for optimal growth	Remark
Halophiles	*Halobacterium salinarium*	High salt (3-4M) NaCl	Aerobic; Has a modified purple membrane at low oxygen concentration.
Thermophiles	*Desulfurococcus*	Optimum temp. 85°C	Obligate thermophilic.
Psychrophiles	*Methanogeni-umboonei*	-10 to - 20°C	Recovered from polar region / deep sea.
Alkaliphiles	*Bacillus halodurans*	pH 9-11	Many have Bacillus morphology.
Piezophiles	*Methanococcus-jannaschii*	Pressures above 300 atm.	Ocean floor / some are also thermophilic.
Thermoa-cidophiles	*Sulfolobus Picrophilus*	70-80 °C; pH 2-3 Optimum temp. 60°C; optimal pH 0.7	Cell wall has lipoprotein and carbohydrate; Found in hot acid springs

Their cell wall is chemically different from that of bacteria. It lacks both muramic acid and D-amino acids. They are either Gram positive or Gram negative. Some Gram-positive archaea have pseudomurein (*Methanobacterium*) while in others the wall is made up of heteropolysaccharides (*Methanosarcina)*. The Gram-negative archaea have a layer of protein (*Methanococcus)* or glycoprotein (*Sulfolobus)* covering the plasma membrane. None of them have peptidoglycan (murien) as their wall material.

A very significant adaptation for survival under harsh conditions is seen in their membrane lipids. They are characterised by long chain branched hydrocarbons attached to the glycerol backbone linked via more stable ether linkage rather than ester linkages (Fig.1.5). Two glycerols may be further joined to form tetra-ethers as in *Thermoplasma.* In addition, they have other polar lipids (phospholipids and glycolipids) and non-polar lipids derived from squalene. The membrane may be organised as bilayer, monolayer or a mixture.

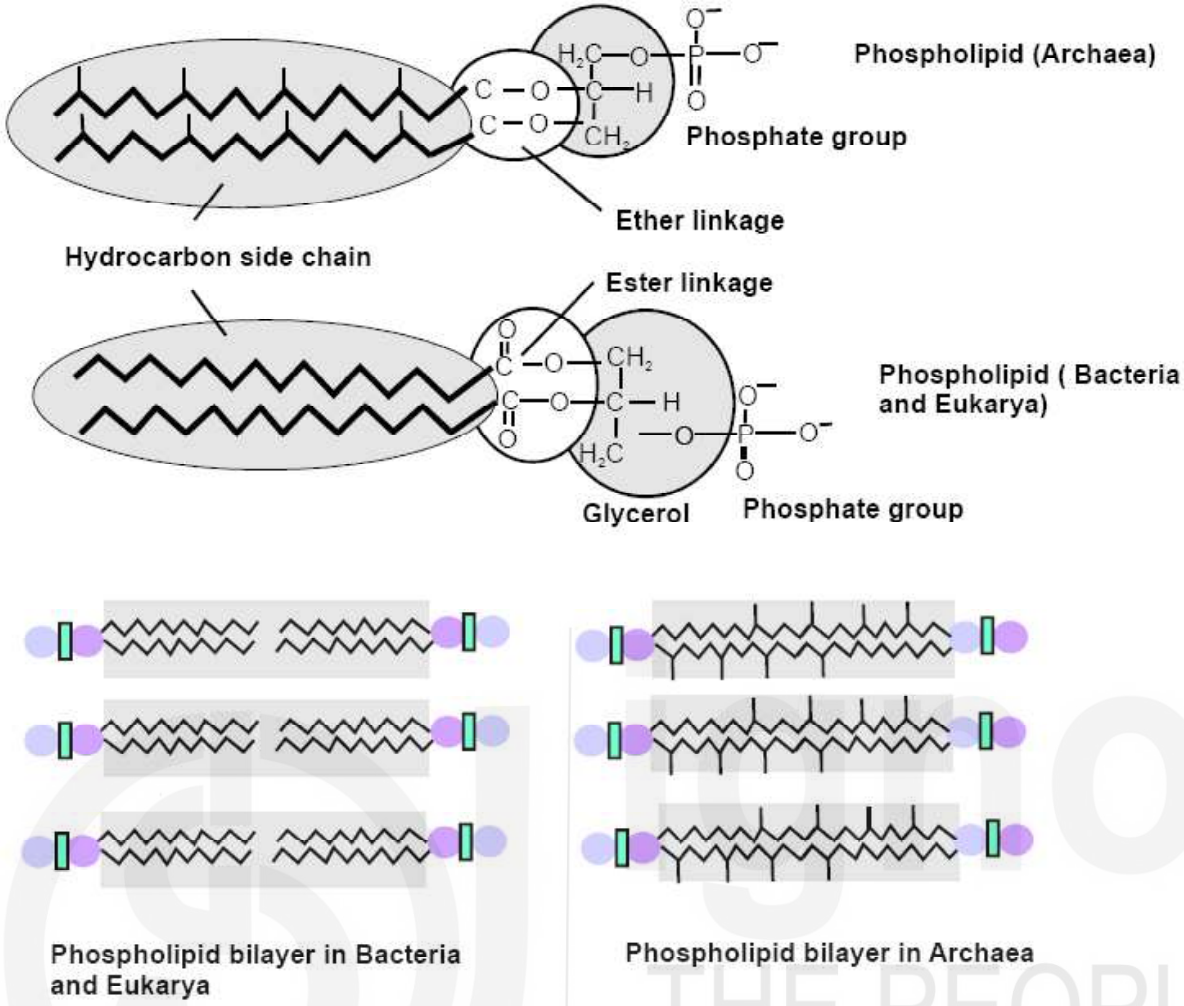


Fig. 1.5: Unusual Ether linked lipids of Archaea.

1.3.2 Eubacteria or Bacteria

Eubacteria represent the second major group of prokaryotes. The prefix 'Eu" signifies 'true" and so we are now going to study true bacteria. They are simpler morphologically and in their internal architecture than eukaryotes. They are unicellular and their cell size is generally small but they do how variations in size. Some are as small as viruses whereas others are larger than an average eukaryotic cell.

Bacteria are generally roughly spherical (coccus) or elongated rods (bacillus). They generate a lot of variation from these two basic shapes. The rounded spheres may exist either singly or have different arrangements depending on the number of planes the cells divide and then they remain adhered to one another (Fig.1.6).

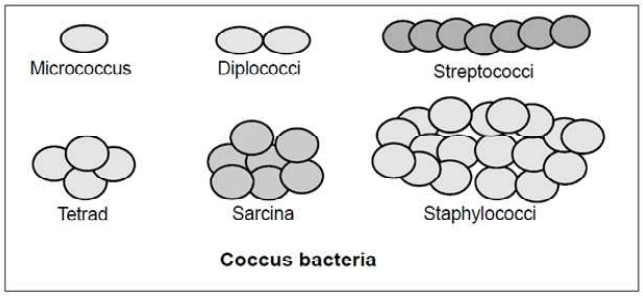
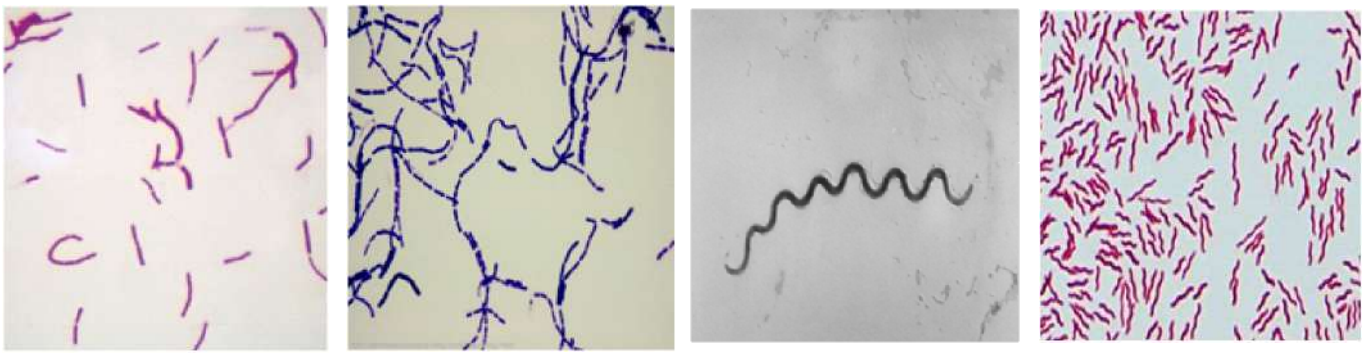


Fig.1.6: Variation in shapes of Coccus bacteria.

The rod-shaped bacillus differs in their length, shape of the rods and they also exist either singly or in chains with two or more remaining together after division. Some of them are also curved or spiral shaped (Fig.1.7).



Bacillus subtilis **Streptobacillus** **Treponema palladium** **Vibrio cholerae**

Fig. 1.7: Representative bacterial rod (bacilli) variations.

Bacteria are ubiquitous (present everywhere) in nature and they have been isolated and cultured from almost every ecological niche. We will study in detail the structure of a typical bacterial cell. The Fig.1.8 is a schematic depiction of a bacterial cell.

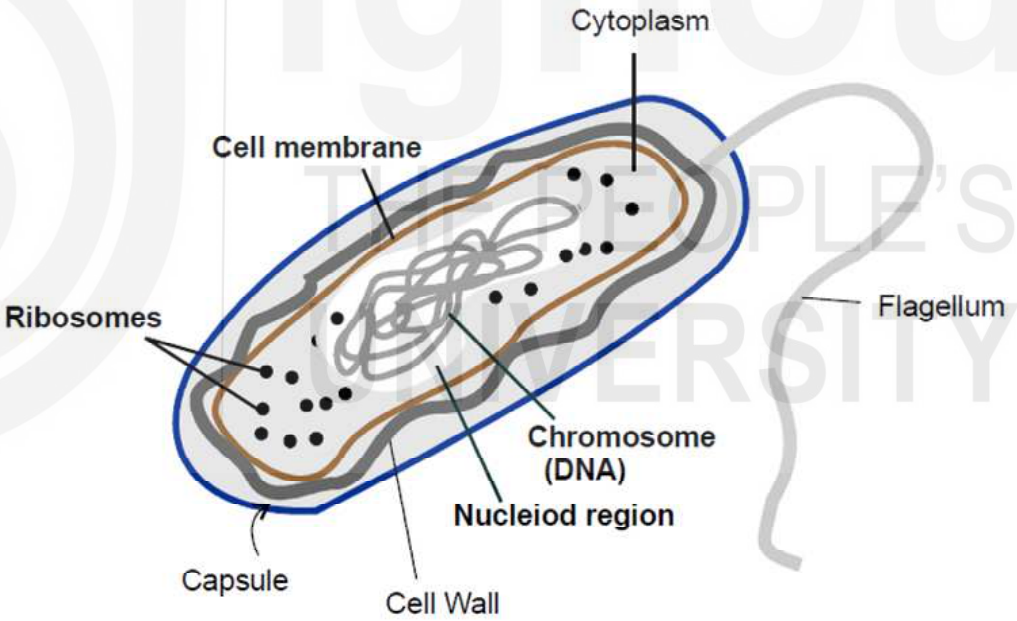


Fig. 1.8: A schematic diagram of a bacterial cell.

A typical bacterial cell has a complex cell wall. They have only a plasma membrane underneath it and are characterised by the absence of membrane bound organelles. Their genetic material is localised in an irregularly shaped region called the **nucleoid.** The cytoplasm has ribosomes (70s) and inclusion bodies that store both organic and inorganic substances and help to reduce the osmotic pressure of the cell. The latter may be enclosed in a membrane or lie free in the cytoplasm. Some bacteria have a slime layer or a capsule outside their cll walls. They may have one or more flagella.

In the absence of typical organelles, bacteria do possess membrane bound invaginations of the plasma membrane that are called **mesosomes.** They are

more prominent in Gram positive bacteria as compared to the Gram-negative bacteria.

The genetic material of most bacteria is a single **circular double stranded DNA**. Some do have linear chromosomes or more than one chromosome. In addition, many bacteria harbor **plasmids** that confer adaptive advantages such as antibiotic resistance or even make them pathogenic. Bacteria multiply by binary fission and have a short generation time. An E. coli cell divides every 20 minutes under optimal conditions.

Bacterial cell wall has **peptidoglycan (murein**) as a common constituent in all bacteria. The variations in cell wall composition among bacteria form the basis of the Gram reaction. The Gram-positive bacteria have a multilayered peptidoglycan and they also have negatively charged sugar phosphate polymers (teichoic acids). The Gram-negative bacteria have a thin peptidoglycan wall (<10% of wall weight) followed by an outer membrane made up of lipopolysaccharides (LPS).

Let us now study the role(s) of major prokaryotic structures. Table 1.2 is an overview of the structures generally found in bacteria along with their function(s).

Table: 1.2: Summary of key bacterial structures and their role(s)

Prokaryotic structures	Role(s)
Plasma membrane	Mechanical boundary/selectively permeable barrier/ senses external signals/nutrient transport.
Nucleoid	Site of chromosome localisation.
Cell Wall	Maintain cell shape/Protect from osmotic lysis
Inclusion bodies	Storage of glycogen, poly phosphate, poly-hydroxy butyrate (PHB) etc/Reduce osmotic pressure (OP)
Ribosomes	Site of protein synthesis
Flagella	Locomotion
Mesosomes	May facilitate chromosome segregation
Capsule / Slime layer	Protection from desiccation/Evasion from immune system.
Endospores	Survival under stress.
Gas vacuoles	Provide buoyancy

Bacteria are extremely diverse in their carbon and nitrogen metabolism and oxygen dependence. They may be obligate anaerobes (*Clostridium pasteurianum),* facultative anaerobes (*Escherichia coli)* or obligate aerobes (*Azotobactervinelandii*). The cyanobacteria such as *Nostoc* and *Anabena* are

interesting examples of obligate aerobes, nitrogen fixers and do carbon dioxide fixation (oxygenic photosynthesis) as higher plants. On the other hand some bacteria are heterotrophs.

We end this section with a note that life without prokaryotes is not possible. Our interdependence is absolute. Most often they are beneficial to us. You are all aware of the **microbial flora** that inhabits the human gut system and it not only protects us from harmful invasions but is a source of nutrients such as vitamins. What will come as a surprise to you is their number. Current estimates suggest the microbial population is at least 10 times greater than the total number of cells in the human body. As you know every good has a darker side. The same is true with bacteria as some of them are also pathogenic.

SAQ 2

a) Fill in the following blanks with correct answer:

i) The most primitive organism on earth is .....

ii) The genetic material (DNA) of prokaryotic cells occurs in form of ..... in the cytoplasm.

iii) Some bacteria form..... to overcome environmental stress.

iv) Thermophiles are bacteria that can survive at ..... temperatures while Psychrophiles can live in..... environment.

v) Prokaryotic organisms contains ..... ribosomes.

b) State whether the following statements are true or false. If false, point out the error:

i) Gram reaction is due to differences in plasma membrane composition of bacteria. [ ]

ii) Biological nitrogen fixation is unique to prokaryotes. [ ]

c) Match the term in column I with that in column II :

Column I	Column II
1. Organisms survive live in extreme conditions	a. human gut
2. Mesosomes	b. bacterial cell wall
3. Obligate aerobe	c. extremophiles
4. Peptidoglycan (murein)	d. Azoto bactervinelandii
5. microflora	e. Plasma membrane invaginations

d) How does the cell wall of archaea differ from those of other bacteria?

1.4 EUKARYOTIC CELL

In this section, you will be introduced to the third major domain that includes all eukaryotes. Plants, animals, fungi and protists (algae, ciliates and protozoa) belong to this group. They can be distinguished from the two subdivisions of prokaryotes primarily by having a well-defined nucleus and many membrane bound organelles. Fig. 1.9 depicts the basic sub cellular organisation of a typical eukaryotic cell.

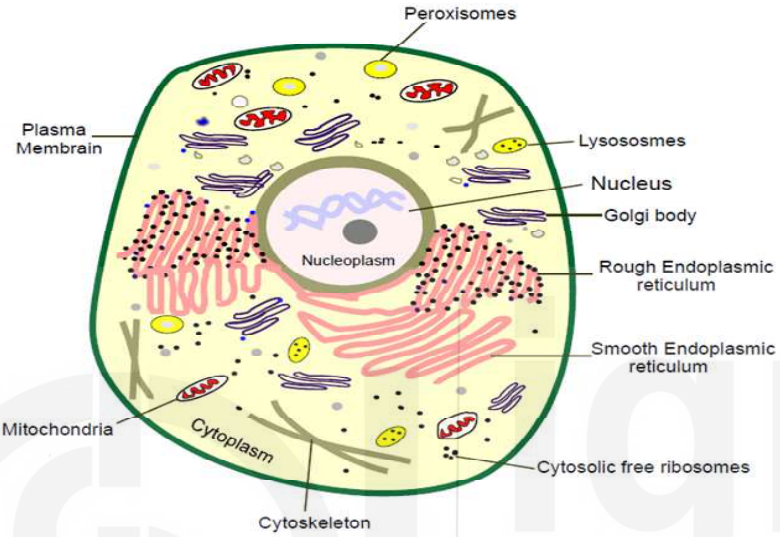


Fig. 1.9: A Schematic diagram of eukaryotic cell.

The average size of a eukaryotic cell is generally larger (10-100 μm) than most prokaryotic cells (1-5 μm). The plasma membrane sets the boundary of the cell as in prokaryotes. Plants in addition, have a rigid cell wall that is made up of cellulose.

The **nucleus** is surrounded by the nuclear membrane and the nucleoplasm has the genetic material and one or more **nucleoli.** The genetic material is complexed with basic proteins such as histones and is segregated into multiple **linear chromosomes**. Most of them are diploid for major part of their life cycle. They divide by either mitosis or meiosis.

A eukaryotic cell is compartmentalised by many **membrane bound organelles** that include endoplasmic reticulum, lysosomes, peroxisomes, mitochondria (in aerobes) and chloroplasts (in plants). Apart from these organelles the cytoplasm has many ribosomes (80s) and protein degrading complexes called proteasomes. The organelles have evolved to perform specific functions. Some like the mitochondria and chloroplast are **semi-autonomous** as they also possess their own DNA. Ribosomes and proteasomes are conserved structures and both appeared earlier in evolution and are therefore, shared by all three groups.

They have a well organised internal **cytoskeleton** made up of proteins. It is a very dynamic structure that can readily reorganise itself in response to the needs of the cell. It performs important role in cell division, control of cell shape and movement of substances within the cell.

Eukaryotes can be broadly classified into **plants and animals**. They can be either unicellular or multicellular. Most unicellular eukaryotes belong to the

kingdom protista. The examples in Fig. 1.10 include both unicellular and multicellular plants and animals. It is a general misconception that prokaryotes are unicellular while eukaryotes are multicellular. You can look for more such examples around you.

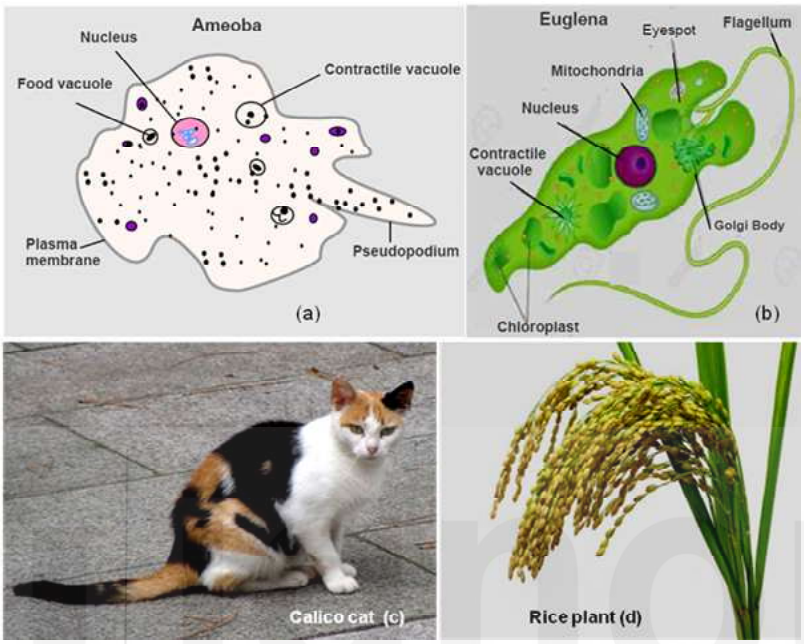


Fig. 1.10: Unicellular (a, b) and multicellular eukaryotes (c, d).

All eukaryotic cells are not morphologically identical. There are about 200 types of cells in the human body which make up the tissues and organs and their shape and size may vary with the functions they perform. For example, red blood cells are about 7.0 μm and the nerve cells are the longest among the human cells. The shape and sizes of representative human cells types are given in Fig. 1.11. Each of these cell types can associate with the right kind of cells to form the tissues and organs of complex multicellular organisms and perform specialised roles(s). The similar theme also applies to plants. The sequential levels of organisation can be represented as follows:

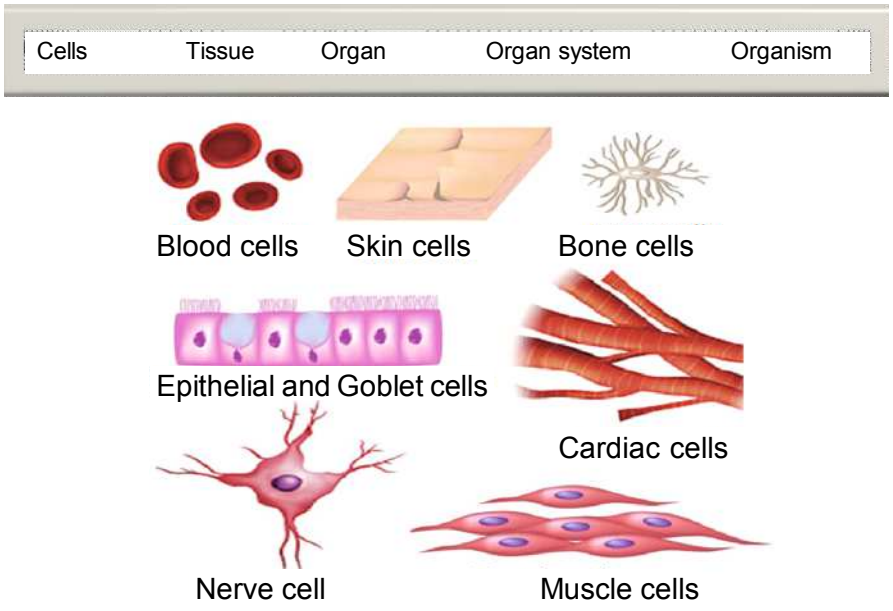


Fig. 1.11: Variation in the shape and size of human cells

Now we will proceed to have an overview of the role performed by various eukaryotic structures. Some of them you would have studied in your class XII biology course. Table 1.3 enumerates the role(s) of the major eukaryotic structures. It is worth noting that all cells do not possess all the listed structures. The next section will highlight some of the differences between plants and animal cells.

Table 1.3: Eukaryotic structures and their role(s)

Structures	Function(s)
Plasma membrane	**Mechanical boundary/ senses signals/ confers selective permeability/ nutrient transport/ Cell-cell and cell-matrix adhesion/**
Cell wall	**Maintains cell shape and rigidity**
Cytoskeleton	**Changes and maintains cell shape; cell division; organisation of the cytoplasm; intracellular movement of cargo.**
Endoplasmic reticulum	**Protein synthesis and transport, modification and protein folding; lipid synthesis.**
Ribosomes (80s)	**Protein synthesis**
Golgi bodies	**Protein glycosylation; Sorting compartment (packaging & sorting of proteins).**
Lysosomes	**Intracellular digestion by acidic hydrolases.**
Mitochondria	**Complete breakdown of carbon by TCA cycle; ATP synthesis by electron transport chain.**
Chloroplast	**Photosynthesis; Starch synthesis.**
Nucleus	**DNA replication and repair, transcription and post transcriptional modifications.**
Nucleolus	**Site of ribosome biogenesis.**
Peroxisomes	**Site for production and breakdown of hydrogen peroxide.**
Vacuole	**Temporary storage and transport; water balance**
Cilia and flagella	**Motility**

Along with this source material watch the video on you tube by clicking on the link given below for a better understanding of the cell structure:
[https://www.youtube.com/watch=URUJD5NEXC8]

1.4.1 Animal and Plants Cells

In the previous section you have already learnt that there are two broad subdivisions of eukaryotes and their roles. Fig. 1.12 is a detailed diagram of a plant and animal cell. When you will carefully examine these two cells, you will realise that most of the sub- cellular structures are common to both such as the endoplasmic reticulum, mitochondria, Golgi complex, lysosomes and peroxisomes. Both have a well-defined nucleus with nucleolus.

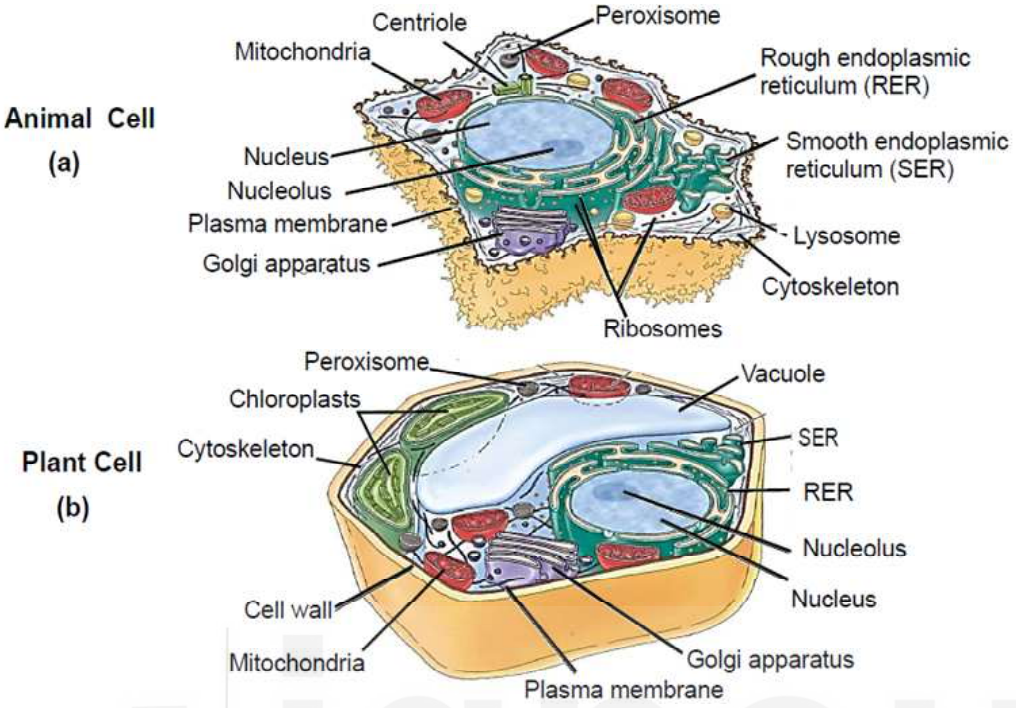


Fig. 1.12: A schematic diagram of an animal cell (a) and a plant cell (b) (Courtesy : M. Cooper, the cell Molecular Approach).

The division of eukaryotes into plants and animal is based on certain differences between them. These differences can also be seen in Fig. 1.12. There are structures present only in plants such as:

- Cell wall
- Chloroplasts
- Vacuoles

1.4.2 Comparison of the Three Major Groups (Domain) of Organisms

Now we have come to the end of our description of the three domains of living organisms. All three groups have many shared characteristics that points to their common ancestry. They have a **plasma membrane** surrounding the cell cytoplasm that provides an internal environment for life processes to occur. The plasma membrane has a bilayer structure (sometimes monolayer in Archaea) made up of lipids and proteins.

Their genetic material is **DNA** and they utilise similar mechanisms for information flow (DNA to RNA to protein). Ribosomes are the site of protein synthesis.

Many catabolic and anabolic pathways are essentially the same in all organisms. The mechanism of ATP (Adonosine triphosphate) generation during respiration and photosynthesis are also well conserved although there are differences in the localisation of the protein complexes among different groups. The energy currency of the cell is ATP.

The three groups have many dissimilarities and their organisation has become more complex since the time they diverged and evolved independently.

Table 1.4 enumerates some key differences between them.

Table 1.4: Difference in structures /processes in the three groups (domains)

Structure / processes	Archaea	Eubacteria	Eukaryotes
Membrane enclosed nucleus and nucleolus	Absent	Absent	Present
Membrane bound organelles	Absent	Absent	Present
Membrane lipids	Long branch chain hydrocarbons attached to glycerol via ether linkage	Long straight or branch chain fatty acids linked to glycerol via-ester linkage.	Long straight chain saturated or unsaturated fatty acids linked to glycerol via-ester linkage.
Cytoskeleton	Absent	Absence of an organised cytoskeleton	Present
Ribosomes	70S (50S and 30S)	70S (50S and 30S)	80S (60S and 40S); 70S in mitochondria and chloroplast.
Cell wall	Pseudomurein / heteropolysac-charides/ proteins	Non cellulosic	Cellulosic ; only in plants
Cell division	Binary fission, fragmentation, budding	Binary fission	Mitosis; meiosis in germ cells
Genetic material	DNA	DNA (generally single circular chromosome)	DNA (multiple chromosomes)

SAQ 3

a) i) Name the sub cellular organelles that are enclosed by a double membrane.

ii) Name the subcellular organelles that involved in the following processes:

a) Photosynthesis (b) Synthesis and breakdown of hydrogen peroxide (c) Synthesis, modification and folding of proteins. (d) Glycosylation and sorting of proteins.

b) **Indicate whether the following statements are true or false. If false point out the error:**

i) Cells of all living organisms have a well-defined nucleus. [ ]

ii) Both animal and plant cells have a cell wall. [ ]

iii) Prokaryotes lack membrane bound organelles. [ ]

iv) All eukaryotes are multicellular organisms. [ ]

v) Only plants release oxegen during photosynthesis. [ ]

vi) Some of the organelles in eukaryotes may contain DNA. [ ]

(c) **Match the items in column I with those in column II.**

Column I	Column II
1. Unicellular eukaryotic organism	a. Mitochondria
2. Multicellular organism	b. Chloroplast
3. Power house of the cell	c. Bacteria/ prokaryote
4. Organelle that converts light energy into chemical energy	d. Plants
5. longest human cell	e. Nucleolus
6. Eukaryotic cell wall	f. *Euglena*
7. Site for ribosomal RNA synthesis	g. Cat
8. Nucleoid	h. Nerve cell

1.5 CELLS AS EXPERIMENTAL MODELS

Our current understanding of biological processes has emerged from the study of model organisms / cells. The organisms used are non-human species that possess some desirable characteristics such as short generation time, availability and are easy to maintain and work with a minimal training. The cost factor is also of paramount importance. This kind of work has much wider implications because of the conservation of major biochemical pathways. Basic principles learned from one kind of cells are generally applicable to others.

A number of model systems have been in use from time to time as no single organism or cell type can have all the desirable features. For instance, it is better to use transparent embryos for observing changes taking place during development. This is one of the reasons why Zebra fish has over taken Drosophila especially for studying very early development. Similarly, small mammals (mouse) have been used to study human diseases or response to drugs due to their genetic and physiological similarities to humans. For some studies cell lines are also useful. Table 1.5(a and b) numerates the salient features of eight model organisms along with the biological processes investigated and important principles established using them.

No doubt many essential pathways are conserved. But we must keep in mind that as we evolve organisms become different from one another by losing some characteristics and acquiring others. Therefore, all that we establish in any model system cannot be extrapolated without documentation.

Table 1.5(a): Characteristics of common model organisms

Model Organisms elucidated	Salient features	Biological processes studied/principles
Escherichia coli 	Unicellular enteric bacteria. Multiplies very fast (divides every 20 min), Small genome / haploid organism. Most strains are non pathogenic Easy to culture	Widely used in genetic engineering Basic mechanisms of gene regulation. Elucidation of the genetic code. Mechanisms of DNA replication/ transcription and translation.
Caenorhabditis elegans (nematode) 	Life cycle is completed in 3 days. It is a hermaphrodite animal with only 959 somatic cells. 0.1-0.3% is also males. Small size and is easy to maintain. Produces about 1000 eggs / day. **Transparent body** makes it easier to trace the fate of cells. **Cultures can be frozen and revived with ease.**	Apoptosis. RNA interference (RNAi). Development. Neurobiological studies. Aging. Congenital heart disease.
Drosophila melanogaster 	Short generation time (10 days at 24^{0}C). Sexually dimorphic. Virgin females are easily identifiable for controlled mating. Larval salivary glands have polytene chromosomes with a consistent banding pattern that allows detection of aberrations.	Development /Genetic control of pattern formation. Biological rhythms (Biological clock); learning and memory. Basic principles of heredity such as gene mapping and sex linked inheritance. Human neurodegenera-tive diseases.
Saccharomyces cerevisiae **(budding yeast)** 	Unicellular eukaryote. **Exists as both haploid** Unicellular eukaryote. **Exists as both haploid and diploid forms.** The products of meiosis are recovered in an ascus (tetrad).	Cell cycle control Mitochondrial genetics. Recombination. Tetrad analysis

Table 1.5(b): Characteristics of common model organisms

Model Organisms	Salient features	Biological processes studied/principles elucidated
***Arabidopsis thaliana* (flowering plant)** 	Short generation time (6 weeks); Small size (10-20cm); Can be both self fertilised and crossed. It produces large number of seeds. Small genome size. A large part of the genome is duplicated.	Development of floral structures Gene regulation. Disease resistance.
***Danio rerio* (Zebra fish)** 	Small fresh water fish. Omnivorous. Easily identifiable males and females. Mating starts at day break. Fertilisation & development is external A **transparent embryo** allows studying development of internal structures. Development is very fast.	Vertebrate development. Human diseases. Biosensor of water pollution. Pigment patterns.
***Zea mays* (corn)** 	Monocot cereal crop. Some chromosomes have "knobs". Cross pollinated in nature. A large portion of the genome is composed of transposons.	Transposable genetic elements QTL mapping. Male sterility. Epigenetics
***Mus musculus* (Mouse)** 	Reproduces rapidly. Closely related to humans. Easy to maintain under lab conditions. Inbred strains are available.	Genetic basis of cancer and testing of potential carcinogens, study of Human diseases such as Parkinson's, Cystic fibrosis, Diabetes and auto immune diseases and drug analysis.

The genomes of the commonly used model organisms have now been sequenced. There is increasing information on the number of genes they encode and their function. A significant number of genes have homology with human genes supporting the use of model systems to address human issues. The Table 1.6 provides genome related information of eight model organisms.

Table 1.6: Genome related information of few model organisms

Model Organisms	Genome information
Escherichia coli	Genome size: 4.64 MB; Chromosome number: 1 No. of genes: 4300 Homology with human genes : 8%
Caenorhabditis elegans **(nematode)**	Genome size: 103 MB; Chromosome number: 2n=12 No. of genes: 20,500 Homology with human genes: 25%
Drosophila melanogaster **(fruit fly)**	Genome size: 175 MB; Chromosome number: 2n=8 No. of genes: 14,000 Homology with human protein genes: 50%
Saccharomyces cerevisiae **(baker's yeast)**	Genome size: 12 MB; Chromosome number: 2n=16 No. of genes: 6144 Homology with human genes: 25%
Arabidopsis thaliana **(flowering plant)**	Genome size: 125 MB ; Chromosome number: 2n=10 No. of genes: 25,700 Homology with human genes: 18%
Danio rerio **(Zebra fish)**	Genome size:1.5x10 billion bp; Chromosome number: 25 No. of genes: 26247 Homology with human genes: 70%
Zeamays **(corn)**	Genome size: 2500 MB; Chromosome number: 2n=20 No. of genes: 32500
Mus musculus **(Mouse)**	Genome size: 2.7 billion bp; Chromosome number: 2n=40 No. of genes: 26,762 Homology with human genes : 99%

1.6 SUMMARY

In this Unit, you have learned that:

- All living organisms are made up of cells. The cell is the basic unit of life that performs all biological functions. The Cell theory is a unifying concept in biology.
- Archaea, Bacteria and Eukaryota are the three domains of living organisms that arose from a common ancestor. The phylogenetic tree of life depicts their evolutionary relatedness. The eukaryotes are more closely related to the Archaea than to Bacteria.

- The presence or absence of a true nucleus and membrane bound organelles are the basis of the classification of cell. A typical prokaryotic cell does not have a true nucleus and membrane bound organelles while the eukaryotic cell contains well defined nucleus and subcellular organelles.
- Prokaryotes are unicellular and simpler organism. They include both the archaeabacteria and Eubacteria. The Archaea are known to survive under extreme conditions and represent the first forms of life in this planet. They have developed various adaptations to withstand extreme environment.
- Unlike prokaryotes, eukaryotes are either unicellular or multicellular. The latter are complex organisms and include all types of present day plants and animals. The plant cells are characterised by the presence of chloroplast, large vacuoles and cell wall which distinguishes them from animal cells. .
- Microbes cell, plants and animal are widely used as an organising model in the experimental research for understanding the biochemistry of cells at molecular level. Such studies are also useful in drug development and in the study of human diseases.

1.7 TERMINAL QUESTIONS

1) Why the cell is called the basic unit of life?
2) Indicate two differences between the cell wall of Gram positive and Gram negative Eubacteria.
3) What are extremophiles? Give two examples along with the conditions in which they live.
4) In which ways do archael membrane lipids differ from that of true bacteria and eukaryotes?
5) Give examples of prokaryotes that are:
   i) Nitrogen fixers
   ii) Obligate aerobes.
   iii) Carbon dioxide fixers (photosynthesis)
   iv) Capable of surviving both in the presence and absence of oxygen.
6) Enlist the Major differences between prokaryotic cells and eukaryotic cells?
7) Enlist the role(s) of the following:
   i) Nucleolus
   ii) Peroxisomes
   iii) Inclusion bodies
   iv) Capsule / slime layer
8) Name the archaea bacteria that can survive (i) in the hot environment, (ii) presence of methane and (iii) in cold temperature.

9) List the organelles responsible for following functions :

(i) DNA Replication; (ii) Energy production;

(iii) Protein transpot; (iv) Digestion of foreign molecules

10) Name the model organisms are widely used for drug development and developmental biology? Highlight the reasons for choosing them.

1.8 ANSWERS

Self-Assessment Questions

1. (i) (a) False (b) True (c) True (d) True

(ii) Please refer subsection 1.2.1

2. a) (i) Archaeabacteria; (ii) Nucleoid (iii) Endospores

(iv) High temperature (>85^{o}C) and in very low temperature (–10^{o}C or even lower)

(v) 70S ribosomes.

b) i) False ii) True

c) 1-c, 2-e, 3-d, 4.-b 5.-a

d) Consult sub section 1.3.1 for details.

3. a) (i) Mitochondria; chloroplast; nucleus; etc'

(ii) (a) Chloroplast (b) peroxisomes

(c) Rough Endoplasmic reticulum (d) Golgi Body

b) (i) False (ii) False (iii) True (iv) False

(v) False (vi) True

c) 1. f; 2. g; 3. a; 4. b; 5. h; 6. d; 7. e; 8. c

Terminal Questions

1) The cell is the smallest structural and functional unit of all living organisms. Cells in unicellular organisms are capable of performing all the life processes. They store genetic information that can replicate and pass on the information to descendent cells and it also directs the cell to synthesise biomolecules needed for all cellular activities. The multicellular organisms assemble and generate complex structures from this basic unit, the cell.

2) Gram positive bacteria have a multilayered of peptidoglycan cell wall with negatively charged sugar phosphate polymers (teichoic acids) while the Gram-negative bacteria have a thin peptidoglycan wall and its outer membrane is made up of lipopolysaccharides (LPS).

3) Extremophiles are archaea bacteria that can survive under extreme environmental conditions Please refer to Table 1.1 for examples.

4) The Archael membrane have long branch chain hydrocarbons attached to glycerol via ether linkage which differentiate them from the membrane composition of true bacteria and eukaryotes. Refer to section 1.3.1 for more details.

5) (i) *Klebsiella pneumoniae* *(ii) Azotobactervinelandii*
(iii) Anabena *(iv) Escherichia coli*

6) Refer to Table 1.4.

7) (i) Ribosomal RNA (rRNA) synthesis and ribosome biogenesis
(ii) Synthesis and breakdown of Hydrogen peroxide
(iii) Storage of both organic and inorganic substances
(iv) Protection from desiccation / Evasion from immune system

8) (i) *Thermus aquatics* survives at high temperature
(ii) *Methanococcusjannaschii* lives in the presence of methane
(ii) *Psychrobacter* lives at low temperature. / Any other examples.

9) (i) Nucleus for DNA Replication
(ii) Mitochondria for Energy production
(iii) Golgi body for proteintargeting
(iv) Lysosomes for intracellular digestion.

10) Mouse is an experimental model widely used for studying the response to drugs because its physiology and biochemistry is similar to that of the humans. *Drosophila melanogaster/ Zebra fish are* widely useful for studying the development biology of eukaryotic organisms. Consult Table 1.5a and b for their suitability.

1.9 SUGGESTED READINGS

1. The Cell: A Molecular Approach (2009) 5th ed., Cooper, G.M. and Hausman, R.E., ASM Press & Sunderland (Wasthington DC), Sinauer Associates, MA, ISBN: :978-9-8793-300-6.

2. Molecular Cell Biology (2012) 7th ed., Lodish. H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D. and Darnell, J., W.H. Freeman & Company (New York), ISBN : 13:978-1-4641-0981-2/ISBN : 10: 1-4641-0981-8.

3. Molecular Biology of the Cell (2008) 5th ed., Alberts, B., Johnson, A., Lewis, J., and Enlarge, M. Garland Science (Princeton), ISBN : 0-8153-1618-4/ISBN : 0-8153-1620-8.

4. Cell and Molecular Biology, (2011) 8th Edition, E.D.P. De Roberties and E.M.F. De Roberties, Jr. Lippincott Williams and Wilkins.

5. Along with this source material watch the video on you tube by clicking on the link [https://www.youtube.com/watch?v=URUJDSNEXC8].

UNIT 2

OPTICAL MICROSCOPY

Structure

2.1 Introduction
Expected Learning Outcomes

2.2 Principle of Light Microscopy

2.3 Cell Fixation

2.4 Brightfield and Dark field Microscopy

2.5 Interference and Phase Contrast Microscopy

2.6 Fluorescence microscopy

2.7 Confocal Microscopy

2.8 Summary

2.9 Terminal Questions

2.10 Answers

2.11 Suggested Readings

2.1 INTRODUCTION

In unit I you have learned about the basic unit of life, the Cell. You must be studied from your school biology course that cells are not visible through the naked eye. We need microscopes to observe their morphology and internal organisation. The advancements in cell biology have paralleled with improvements in microscopy. The developments in the area of microscopy have paralleled with our understanding of cells. Therefore, the focus of Unit II and III in this block is microscopy. The information in the two units is subdivided depending on whether it is an optical or electron microscopic technique. In this unit you will be introduced to optical (light) microscopy that uses visible light to observe specimens.

We begin this unit with the basic principle of Light microscopy. Here you will learn about resolution (resolving power), magnification and contrast which are important consideration for getting good quality image. You will also learn about cell fixation methods for sample preparation prior to light microscopic observation. Then we move on to different types of light microscopy and understand their principles and applications. Routine microscopy work generally involves simple light microscopy. In this case transmitted light from the sample helps in its visualization. This method is called bright field microscopy as the specimen background appears bright. Instead of transmitted light, imaging may also be done using of incident light. In that case the background appears dark and the object is brightly illuminated (darkfield microscopy). Thereafter, you will learn about the working principle and application of Interference and Phase Contrast Microscopy. The importance of

fluorescence microscopy and confocal microscopy in the Biomedical Science are also discussed. As you know magnification obtained using light microscopy has a limit. For magnification beyond the limit of light microscopy, electron microscopy is used about which you will learn in Unit 3.

Expected Learning Outcomes

After studying this unit, you should be able to:

- explain the basic principle of Light microscopy;
- indicate the methods and purpose of cell fixation;
- describe the principles and applications of :
  - Bright-field and Darkfield Microscopy
  - Interference and Phase Contrast Microscopy
  - Fluorescence Microscopy
  - Confocal Microscopy

2.2 PRINCIPLE OF LIGHT MICROSCOPY

A microscope is an instrument that has lenses to magnify objects and produce visual or photographic images of minute objects. It is an indispensable tool because of the inherent limitations of the human eye to observe minute objects around it. An unaided human eye is unable to focus objects that are brought closer than 25cm (least distance of distinct vision, LDDV) and therefore anything that is approximately smaller than 0.1mm is not visible.

The cells of most organisms are smaller than what the human eye is capable of seeing. The field of microbiology and detailed understanding of cells of all organisms began with the invention of the microscope. The history of early microscopes and some leads in the discovery of the microbial diversity are given in Box 1.

Box 1: Early history of microscopes

- **The early microscopes were of two kinds: simple microscopes and compound microscopes**.

The **simple microscopes** had a single lens of very short focal length and were capable of high magnification. They were not very different from the ordinary magnifying glasses that have been known to mankind from time immemorial.

The **compound microscope** has two sets of lenses- the objective and the ocular (eyepiece). It has a greater intrinsic power of magnification. The image seen by the eye is the product of the magnification of the two lenses.

The first compound microscopes were made by two Dutch spectacle makers ***Zacharias Janssen*** **a**nd ***Hans Janssen*** in the 1590s.

The credit of making the first developed microscope goes to ***Antonie van Leeuwenhoek*** (1632-1723), a Dutch draper. He made his first microscope to observe the quality of cloth that he manufactured. Later he went on to observe many microbes using his microscopes. He was the first to describe many bacteria, yeasts and other microorganisms found in natural surroundings. Interestingly he used a simple microscope that had a tiny biconvex lens and some of them could magnify from 200- 300x.

Most of Leeuwenhoek's contemporaries and successors used compound microscopes. Although they were superior to the simple microscopes but the ones available in the 17th and 18th century suffered from **optical defects** such as spherical and chromatic aberrations. They were therefore less effective than the simple microscopes.

The English Scientist ***Robert Hooke*** further developed Leeuwenhoek's findings using a **primitive compound microscope**. In 1665 he observed cork cells and used the term cells to describe them.

- **It is important to emphasize that some of the greatest earlier microscopic discoveries were made with simple microscopes.**

In the 19th century (more than 200 years after the invention of the microscope) the problem of optical aberrations was addressed and slowly compound microscopes overtook the simple microscope in generating good quality images.

Today, microscopy is an important aspect of discovering and characterizing life processes. Since the time of Leeuwenhoek, the field of microscopy has seen many technical advances which will be described later in this unit.

Most present-day microscopes are compound microscopes so we will now study the basic parts of a compound microscope and the principle of light microscopy. Fig. 2.1 is a schematic representation of a compound microscope.

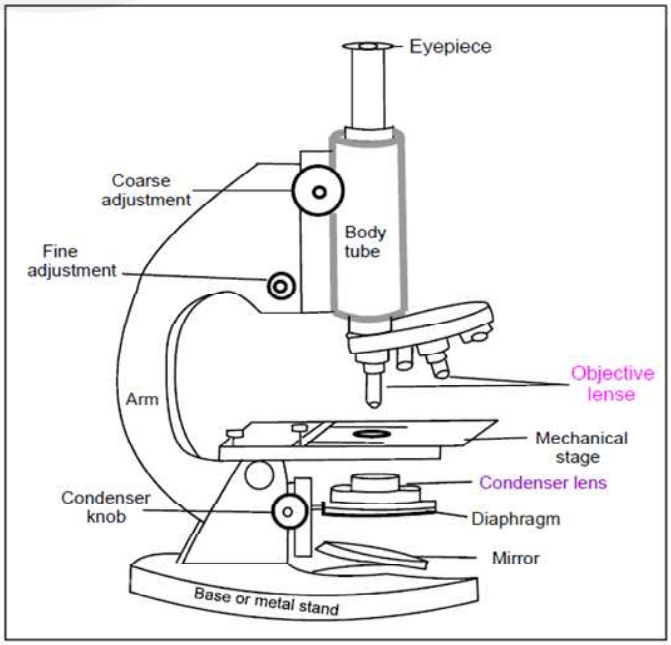


Fig. 2.1 : A schematic illustration of Compound microscope.

In a compound microscope there are **two types of magnifying lenses**, namely the objective and the ocular (eyepiece). The **objective lens** is at the lower end of the body tube close to the specimen. It collects light coming from various parts of the specimen and produces a real, inverted magnified image inside the body of the tube. It also plays an important role in producing a high resolution clear image. The eyepiece is fitted at the other end of the body tube and is farthest from the specimen. The **eyepiece** further magnifies the real image produced by the objective. The observer sees this magnified image and it appears as it were near the base of the microscope. The distance from top of the microscope tube to where the objective fits into the nose piece is usually 160mm. It is possible to alter the resultant magnification by changing the objective and ocular lenses.

The microscope is equipped with two sets of **knobs for coarse and fine adjustments**. They also permit adjustment of the stage position. The specimen slide is placed on the **stage** that has an opening in the centre to allow light to pass though from the light source (built in lamp). Most microscopes can hold the slide in position and it can be moved in all possible directions while making observations.

The **condenser** is located below the stage. It has lenses that help to concentrate the light coming from the lamp onto the sample. The condenser can be moved up and down. As a part of the condenser is the **diaphragm** that can be opened or closed. Both the condenser and the diaphragm are important in providing good illumination.

The microscope tubes may also be **binocular or trinocular**. The binocular tube produces less eye strain and has become more popular. In these microscopes the horizontal distance between the two eyepieces is adjustable to fit the inter pupil distance of the eyes of different investigators. A trinocular microscope has one of its tubes connected to the camera. In this it is possible to both view the specimen and to click a picture for record by simply flipping the light and redirecting it to the camera.

In order to generate good quality magnified image three most important parameters are:

- **Magnification:** The magnification power of a microscope is its ability to produce an enlarged or magnified view of an object to the eye.
- **Resolution:** The ability of a microscope to allow distinction between two close but separate points in the specimen is its resolution.
- **Contrast:** The object must possess certain degree of contrast with the surrounding medium in order to be seen clearly through the microscope

The Fig. 2.2 explains how the combination of the two lenses form a **magnified image** of the object in a compound microscope. The sample is placed on the stage and illuminated using a light source. The condenser helps to direct most of the light from the source to fall on the object. The

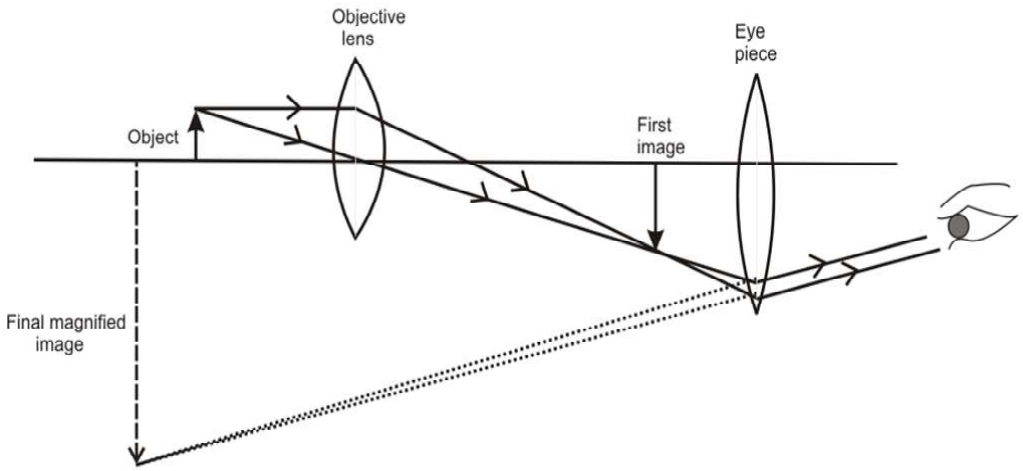


Fig. 2.2 : Image magnification in a compound microscope

objective lens produces an image of the object while the ocular lens (eyepiece) further magnified it enabling us clearly see very small further microscopic objects through the eyepiece. For example, if you use a combination of a 4X objective lens and a 10X ocular lens, then the resultant magnification = (4X10) X = 40X.

The quality or clarity of any image depends upon maximizing the distance between images of any two points of the image. This will allow you to clearly distinguish between those two points. At any given magnification, the **resolving ability** of the microscope would determine how clearly you can see and distinguish between two such close points.

Resolving power of a microscope is directly proportional to the wavelength of light used and inversely proportional to the numerical aperture of the microscope. These values are related by the **Abbe's equation** (including Rayleigh's correction) below.

$$\textbf{Resolving power (d)} = \frac{\mathbf{0.612\,\lambda}}{\mathbf{n \sin \alpha}}$$

Where, λ = wavelength of light used

n = refractive index of medium between the specimen and objective lens

α = half aperture angle in radians.

n sin α = numerical aperture (NA) of objective,

From the above equation you can clearly understand that better resolution is achieved by either changing the wavelength or numerical aperture. This formula of resolving power is used to calculate the resolution of an image in the light microscopy.

In a compound microscope **wavelength of light** that illuminates the sample ranges from 400 to 700 nm and this in turn limits the resolution obtained. The shorter is the wavelength of light, the finer the detail revealed by the objective lense. The best compound microscope cannot resolve parts of a specimen that are closer together than 200nm.

Numerical Aperture (NA): It is a measure of the resolving power of a microscope objective. It is equal to the product of the refractive index of the medium (n) in front of the objective and the sin of the angle (sin α) between the outermost ray entering the objective and the optical axis. To know more about numerical aperture,click this link https://www.microscopyu.com/microscopy-basics/numerical-aperture

Up to a certain limit, an increase in numerical aperture of the objective lens increases resolving power. The value of numerical aperture can be further increased by filling the space between the specimen and the objective with oil as it has a higher refractive index than air. With oil immersion lenses, the numerical aperture can be increased to a value as high as 1.4.

So now we understand how to achieve good magnification and resolution. However, even at very high magnification and resolution, the image quality will be unsuitable for analysis if the contrast is poor.

Finally, we need to generate contrast such that less light is transmitted through the sample than through the medium. The decrease in light transmission is caused due to light absorption by the cells and light refracted out of the optical path of the microscope by a difference in refractive index between the cell and the medium. The degree of contrast of the image can be increased by staining the specimen and / or by optimizing the optical settings in the microscopes.

SAQ 1

a) Explain how the magnified image of an object is obtained in a compound microscope.

b) Calculate the resolving power of the light microscope if NA = 1.4 and λ =400nm or 500nm.

c) What is the magnification of the optical microscope if you are observing a specimen with a 40X objective lens and a 10X ocular lens?

2.3 CELL FIXATION

In the preceding section you have leant about the basic principle of microscopy and the working of a compound microscope. However, before we start observing specimens, it is necessary to fix cells or tissues so that we can preserve them in a near lifelike condition. Cell fixation prevents autolysis and putrefaction of the specimen and also protects the tissue / cells during any further processing steps.

Cell fixation can be done either by **physical or chemical** means. Physical fixation is carried out by increasing (heating) or decreasing (cryopreservation) the temperature whereas chemical fixation depends on the use of either organic solvents or cross linking agents. Generally chemical fixation methods are used to preserve most specimens.

The **organic solvents** include alcohol and acetone that cause removal of lipids; dehydration of tissues; denaturation and precipitation of proteins; changes in protein conformation and their solubility. They are also referred to as **coagulants** based on the effect produced. The dehydrating effect of ethanol can be reduced by the swelling effect of acetic acid and they are together used in some cytological fixatives.

The **cross linking methods** uses aldehydes (such as paraformaldehyde, glutaraldehyde) or osmium tetroxide that reacts with proteins and other biomolecules by forming intra- and inter molecular cross links. The covalent bonds result in a better retention of cellular architecture than organic solvents. The rigidity imparted to cells by cross linking is an added asset in sectioning fragile tissues. Fixing kills the cells immediately but allows us to visualize its morphology and internal structures that were present just before fixing. This is helpful because once the cells are fixed then the investigator can observe them at ease. Fixing also permeabilises cells. This facilitates visualizing cellular proteins or other molecules by allowing antibodies/stains such as 4, 6 - diamidino-2-phenylindole (DAPI) for DNA, to enter the cells with easily and bind to their specific targets. Stains give different colours and color density and increase the optical contract between different components of a specimen

There is **no ideal fixative** and it is advisable to work out the best fixative for your sample. Some of the variables that can be standardised are concentration of the fixative; time of exposure, temperature and rate of penetration. Wherever feasible choose a non toxic substitute.

We must keep in mind that fixed cells (dead cells) are not suitable to look at the dynamics or time dependent live changes in cellular processes. Only live cell imaging techniques allows us to undertake such studies.

SAQ 2

a) What is the advantage of fixing cells?

b) Match the term in column I with that in column II

	Column I		Column II
1.	Physical method	a.	binds to proteins
2.	Cross linking methods	b.	dehydration solvent
3.	Alcohol	c.	Chemical fixation
4.	Staining dye	d.	increasing and decreasing temperature
5.	Paraformaldehyde	e.	DAPI

2.4 BRIGHTFIELD AND DARKFIELD MICROSCOPY

Once we are ready with our samples to microscopic examination, the first important consideration that needs deliberation in the choice of the light source for illumination of the sample. The compound microscope described in section 2.2 is a **brightfield microscope** because it forms a dark image against a brighter background. In this the condenser concentrates and transmits the light directly through the specimen. In transmitted light microscopy, an image is formed by undiffracted light interfering with diffracted light from the specimen. A less transparent part of the sample would therefore appear dark against the bright background while more transparent parts will appear less dark and completely transparent part will have the same brightness as the background and therefore may be indistinguishable.

We must remember here that most biological samples are quite transparent. Therefore, to enable us to see them more distinctly, we can take the help of

various stains which will reduce the transparency of the sample and create contrast between the background light and the sample. Bright field microscopy is generally suitable for stained biological samples. Some of the stains routimely used are given Table 2.1.

Table 2.1: Stains commonly used for animal and plant tissue

Stain	Suitable for	Color
Hematoxylin	Nucleus	Purple
Eosin	Cytoplasm and plasma membrane	Pink to red
Methylene blue	Cell nucleus and bacteria	Blue
Toluidine blue	Nucleus of plant cells (in mitosis)	Blue
Iodine	Xylem and Starch content in plant cells	Blue-black
Leishman/Giemsa/ Wright's	Blood and analysis	Violet
Safranin	Plant cell nucleus	Red yellow

The other variation for observing transparent samples is called **darkfield microscopy** in which the object is brightly illuminated against a dark background. Fig. 2.3 is a schematic view of bright filed and dark field microscopes. Dark field microscopy prevents direct light from entering the front of the objective and so the microscope detects light scattered (diffracted) from the object. The diffracted light will enter the objective and reach the eye. The condenser is equipped with a dark field stop to block the light. Most often, special bright light sources are required to get a good image. The dark background provides high contrast to see scattered objects and it is useful for observing low magnification outlines of individual cells such as sperms that scatter light very strongly.

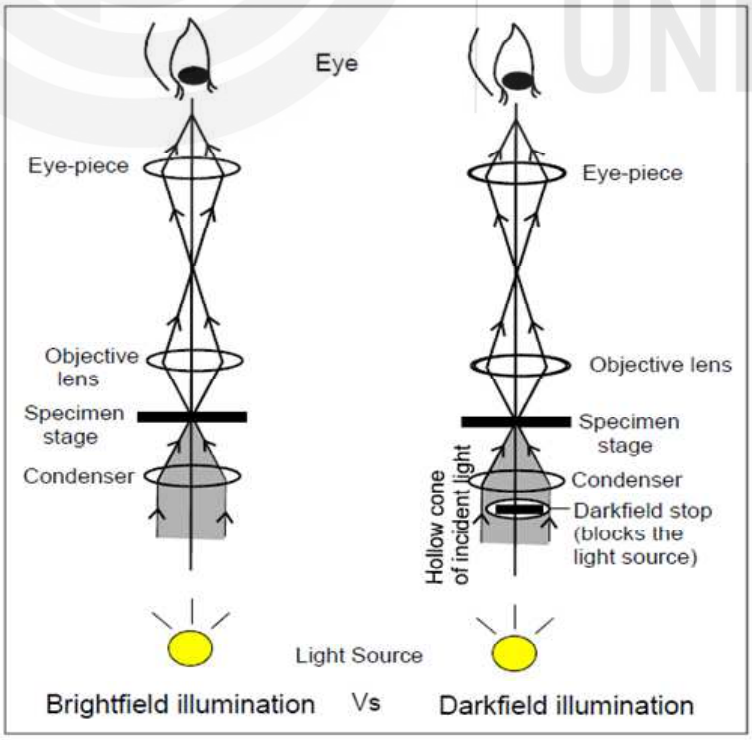


Fig. 2.3: A schematic view of Brightfield and Darkfield microscopes.

Most microscopes can also be used for dark field microscopy such as compound or stereomicroscopes by simply shifting the knob from bright field to dark field. It is used for observing unstained samples especially those organisms that are not easily stained or are distorted by staining. In addition staining kills live cells, dark field microscopy is used to capture live images of bright transparent specimens.

In dark field microscopy resolution is limited by the condition that the NA of condenser is greater than the NA of the objective so that the cone angle of illuminating light is not captured by the objective. However, since dust particles can also scatter light, quality of the dark field microscopic images will suffer from the presence of dust particles in the optical path.

SAQ 3

a) What is the difference in the sample illumination technique used in bright field and darkfield microscopy?

b) Which of the following statements are true or false?
Write (T) for true and (F) for false in the given boxes.

i) Bright field microscope forms a bright image against a dark background. ☐

ii) Scattered light is used to view in the bright field microscopy. ☐

iii) Dark field microscope is used to observe transparent samples ☐

iv) Staining improves contrast between background light and the sample. ☐

v) Bright field microscope is suitable for observing stained samples ☐

2.5 PHASE CONTRAST AND INTERFERENCE MICROSCOPY

In this section you will study two more optical microscopy contrast enhancing techniques, namely phase contrast microscopy (PCM) and differential interference contrast (DIC) microscopy.


Frits Zernike

Phase contrast microscopy (PCM) was invented by **Frits Zernike,** a Dutch physicist in 1934. As the name suggests it is a contrast enhancing technique that can generate high contrast images of transparent specimens such as living cells, thin tissue slices and sub cellular organelles. It is also possible to observe the dynamics of ongoing biological processes.

In phase contrast microscopy (Fig. 2.4), light produced from a tungsten-halogen lamp is directed through a collector lens and focused on an **annulus (ring**) located in the sub stage phase contrast turret condenser. The light can only pass through this annulus so the light reaching the specimen is like a hollow cone. The light waves passing through the annulus illuminate the specimen and either pass through unretarded (surround waves) or are diffracted and retarded as they interact with different regions of the specimen.

The cell may have regions that are thicker and / have a higher refractive index such as nucleus and cytoplasmic inclusions as compared to other areas that are thinner and have lower refractive index. The extent of retardation of light waves passing through the specimen will be greater in thicker than thinner areas. The undeviated and diffracted light is collected by the objective and segregated by a **phase plate.** The phase objective contains a dark annulus that retard the diffracted waves by ¼ wavelengths making them further out of phase with one another and with undeviated waves. What we see is a combination of constructive and destructive interference that takes place as these light waves meet. The waves of light that are in phase produce a **bright image** while those that are out of phase cancel each other to varying degrees depending on the extent to which they are out of phase and produce images

that differ the in degree of darkness (**grey to black**). Frits Zernike won the 1953 Nobel Prize in physics for inventing this technique.

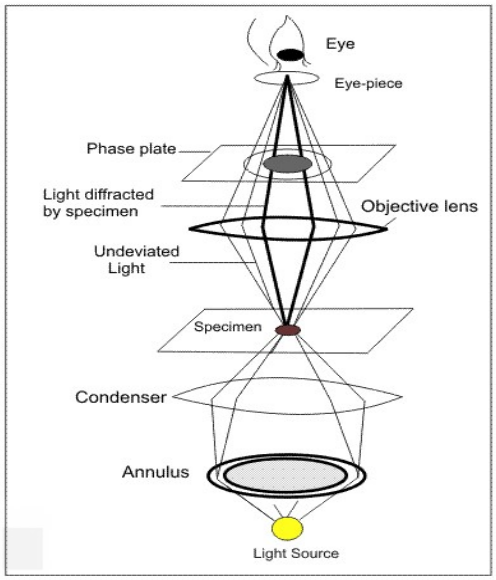
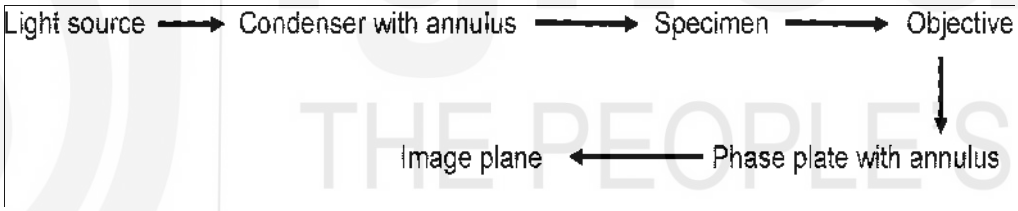


Fig. 2.4: Phase contrast microscope.

❖ **Optical pathways in the phase contrast microscopy are summarised below:**



Differential interference contrast (DIC) microscopy was invented by a Polish physicist **G. Nomarski** and is used to view specimens with poor optical contrast. It is a non invasive technique. Fig 2.5 depicts the optical path of DIC that includes a polarising filter, two double prisms and an analyser.

Georges Nomarski
DIC Microscope

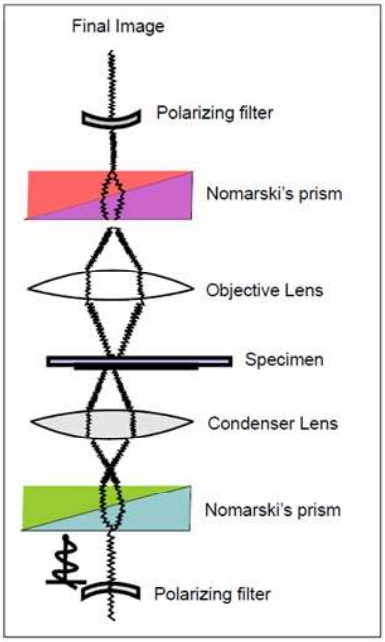


Fig. 2.5: Optical path of DIC microscope

In DIC two beams of light are used. The unpolarised light coming from the lamp first goes through **a polarising filter** that polarising it at 45^0. It is then directed to Nomarski-modified Wollaston prism placed below the condenser. The **prism splits** the entering beam to create **two distinct beams** of polarised light that travel in slightly different directions. These beams do not recombine to cause interference. The distance between the two beams is less than the resolving ability of the objective and is generally referred to as the 'shear' distance.

The two beams then pass through the **condenser lens**, which makes them parallel to one another so that they pass side by side through the specimen. When the beams pass through the specimen their paths are modified due to the difference in thickness and refractive index they encounter. In DIC optical path length gradients are primarily responsible for contrast. As the parallel beams enter the objective they are focused above the rear focal plane and then it enters the second prism that **recombines** the two beams into one polarised beam. The recombination leads to interference. The **second polariser (analyser)** is required to bring the vibration of the beams in the same plane and axis so that interference occurs. The final image is seen through the eyepiece as differences in intensity and colour. The DIC image shows surface details and can produce 3D like image. The cells appear raised from the surface of the slide and even nuclei will appear raised from the surface of the cytoplasm.

SAQ 4

Enlist the main differences between Phase contrast microscopy and DIC microscopy.

2.6 FLUORESCENCE MICROSCOPY

Fluorescence microscopy is a special type of optical microscopy where a fluorescent sample is illuminated with light of shorter wavelength, usually blue or UV light and the emitted photons (longer wavelength) then are measured. It is based on the phenomenon that certain substances emit light energy in the visible region.

Fluorescence has been known to mankind for more than 150 years. The earliest account is of **Sir John F.W. Herschel** in 1845 from water extracts of the bark of Cinchona (source of quinine sulphate). Quinine gives a blue fluorescence. Few years later George Gabriel Stokes (1852) noted that fluorescence always occurred at a longer wavelength than that of the excitation light. This shift in the emission spectrum is called the **Stokes shift** (Fig 2.6).

It is worth noting from Fig. 2.6 that the excitation and emission curves are mirror image of each other except that the wavelengths have shifted and with the increase in Stokes shift values it will be much easier to separate excitation from emission light by using filters. Fig. 2.7 depicts the Jablonski's energy diagrams which are useful in explaining the molecular transitions between excitation and emission of light energy.

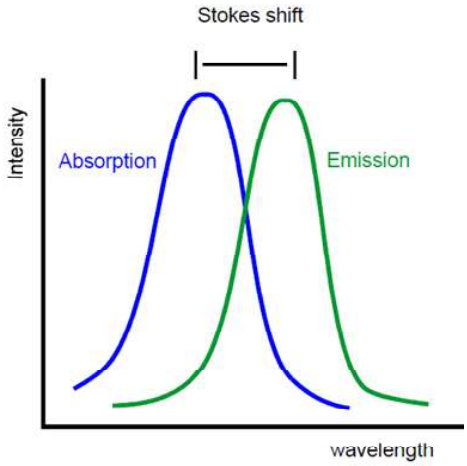


Fig. 2.6: Stokes shift

The use of fluorochromes for biological investigations started around the 1930s. Many specific stains were known by that time that stimulated the development of fluorescent microscopes. Today it has become an indispensable tool in biological and biomedical investigations. Some of the commonly used fluorochromes are listed in the Table 2.1.

Now we will proceed to study the basic components of a fluorescence microscope (Fig.2.8).

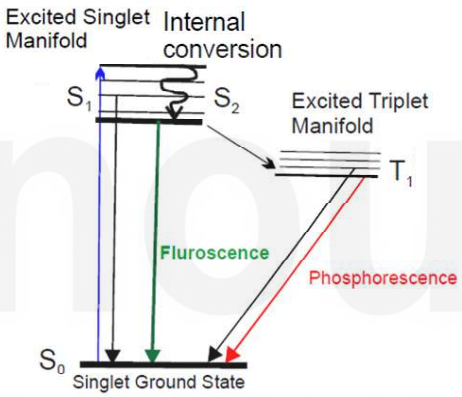


Fig 2.7: Jablonski energy diagram

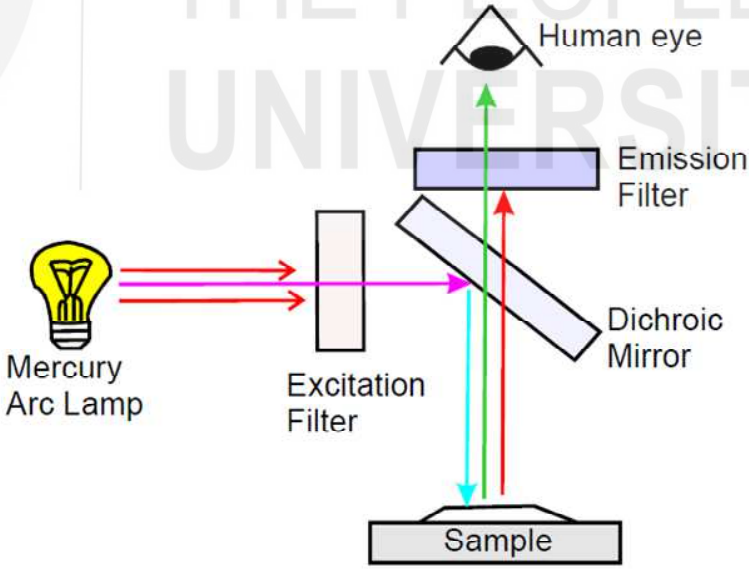


Fig. 2.8: Fluorescence microscope

Basically, a fluorescence microscope is meant to excite the specimen with **bright light** of desired wavelength and separate the weaker emitted light (fluorescence) from the excitation light so that the resultant structure has a high contrast against a dark background.

The basic parts of a fluorescence microscope are:

- **Light source**: A variety of light sources can be used. They have to be powerful light sources because the collection efficiency is less than 30%

and emission signals are weaker. Some of the light sources include mercury or xenon arc lamps and laser light sources.

- **Filters:** Three categories of filters are essentially required. They are excitation, barrier (emission) and dichromatic beam splitters (dichroic mirrors). The filters are high resolution with interference optics. These filters are combined in filter cubes and there are multiple such cubes that can be rotated easily to change filters. The selection of appropriate filters is the key to success.

  The excitation filter transmits only the excitation light of the desired wavelength. Dichroic mirrors are placed in the light path at an angle and they reflect the excitation light from the objective to the sample. A barrier filter removes any excitation light that could otherwise reduce image's contrast.

  Incidentally the objective is designed both to direct excitation light and to function as an image forming light gather. An objective of higher numerical aperture is better for getting a bright image.

- **Detection:** The fluorescent image formed can be visualised through the eyes or camera equipped with sensors to record images in a dark background.

  Some samples are auto fluorescing and other specimens are stained with fluorochromes that fluorescence brightly upon exposure to light of a specific wavelength. A fluorochrome is a fluorescent chemical that can reemit light upon excitation. A fluorochrome s generally excited at wavelength at or near the peak of the excitation curve. Table 2.1 gives the excitation and emission wavelength of some fluorochromes. It is also possible to use multiple fluorochromes to stain different components with different colors.

  An important consideration in the choice of the dye is its **quantum efficiency (QE).** It is defined as the fraction of absorbed photons resulting in emission of fluorescence. Compounds with a higher QE are preferred.

Table 2.1 : Excitation and Emission wavelengths of common fluorochromes

Fluorescent Dye	Excitation wavelength (nm)	Emission wavelength (nm)
Hoechst 33342	343	483
DAPI	345	455
Ethidium Bromide	493	620
Acridine Orange	503	530/640
SYTOX Green	504	523
Propidium Iodide (PI)	536	617
Fluorescein	495	519
TRITC	547	572
EGFP	488	507 (Green)
mCherry	587	610

The major constraint encountered in fluorescence imaging is **photo bleaching**. It is due to an irreversible decomposition of the fluorescent molecule in the excited state due to their interaction with oxygen prior to emission. There are ways available to overcome this problem such as by use of fade resistant compounds (Quantum dots); additions of anti-fade compounds or open the shutter only when required.

Fluorescence microscopy has become an indispensable tool for biologists as it provides high sensitivity, specificity, high contrast and quantitation. It is capable of revealing the presence of a single molecule.

We will end the topic by discussing some of the **applications** of fluorescence microscopy.

- **Live cell imaging**: It is possible to obtain information about cell dynamics. These may include movements and interactions of different biomolecules. Thus, one can measure the rates of physiological reactions, binding constants of bio- molecular interactions, dynamic changes in cellular structures etc. Such studies require time lapse fluorescence imaging where the same area is imaged continuously at regular time intervals and the changes are analyzed.
- **Cell viability:** Viability of cells can be ascertained using vital stains during fluorescence microscopy. Vital stains are dyes that can enter only dead cells and therefore make the dead cells appear fluorescent.
- **Immunofluorescence:** It requires fluorescent labeling of proteins such as antibodies. It may be either direct or indirect labeling. In direct immunofluorescence the labeled antibody (primary) is against the target antigen whereas in case of indirect immunofluorescence the labeled antibody (secondary antibody) is against the primary antibody.

  Most often indirect immunofluorescence is used as it does not require specific labeled antibody against the target antigen and labeled secondary antibodies can be purchased. In addition, the sensitivity of staining is enhanced as multiple fluorochromes labeled antibodies can bind to each primary antibody. It is most often used to localise antigens in tissue sections or sub cellular compartments.

[You can also view to a video lecture by N. Stuurman on Fluorescence microscopy that is freely accessible at ibioseminars.org]

SAQ 5

What is Stokes shift? When EGFP fluoresces, what color of light will be seen?

2.7 CONFOCAL MICROSCOPY

Confocal microscopy or more appropriately confocal laser scanning microscopy (CLSM) includes a family of techniques that essentially have two parts – optical sectioning and confocal. It is advancement over conventional **wide field microscopy** that is known to generate both in focus and out of

focus images (non confocal), especially in case of thicker specimens and this blurring effect eludes the desired details. Confocal microscopy on the other hand produces high resolution in focus (confocal) images.

The basic principle of confocal microscopy was developed by **Marvin Minsky** in 1953 but it took almost three decades for it to become a standard technique. The development of lasers has made this technique to take off and reach the level of refinements we see today. An important issue to address in the successful development of this kind of microscopy was to get rid of the out of focus images. The innovation was simply to place a **pinhole** in the same focal plane as the sample (and so the term 'confocal').

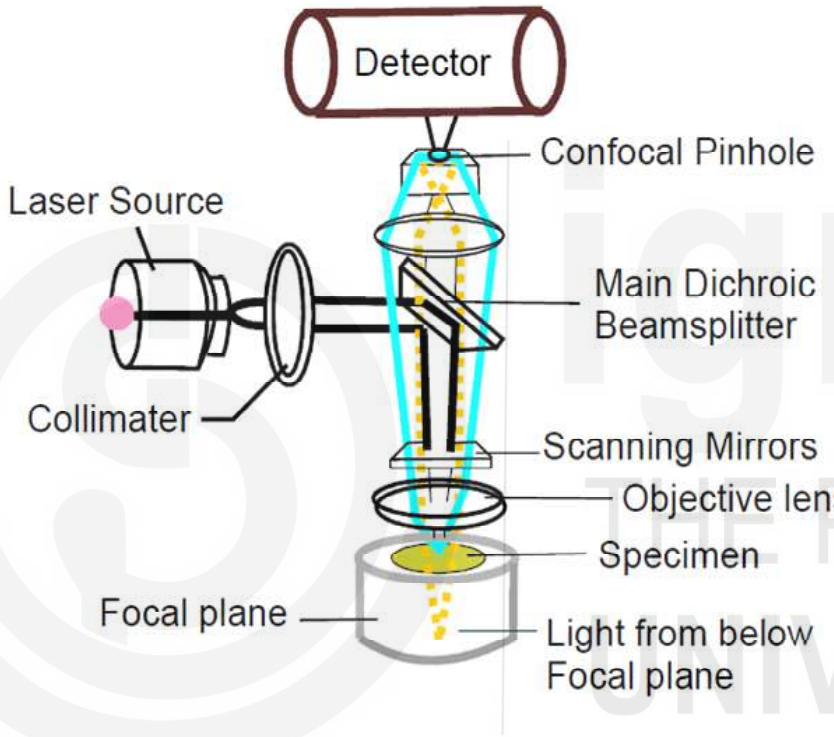


Fig. 2.9: Basic setup of Confocal Microscope

A confocal microscope is made up of the following components (Fig. 2.9):

- The optical system
- The laser light source
- Scanning device
- The detection system

We will now proceed to describe the scanning device and the detection system.

The image is created by **scanning** laser point by point over the fluorescent sample. The intensity is recorded at each point. This method is called ***z-scanning*** as the optical sectioning is along the z-axis of the microscope. As we move the illumination point, the emission point also moves and so it is necessary to check that the pinhole is coincident with it. The stack of two dimensional images generated can be viewed either individually or is

reconstructed by the computer imaging software. The reconstructed image provides a 3D view.

The increased resolution in confocal microscopy is at the expense of decreased signal intensity as only the fluorescent light that passes through the pinhole at the focal point is picked up by the detector. This implies the **detectors** must be sensitive to detect these weak signals and in many cases longer exposures are needed. Since we are scanning spot by spot the detectors must also be fast as the confocal beam spends only a few microseconds at each pixel. Some of the detectors use photomultiplier tubes (PMT).

Applications of confocal microscopy

- It is useful for **3D imaging** of cells in tissues and surface profiling of samples.
- It is a **non invasive** technique involving optical sectioning of thick fluorescent labeled live biological specimens.
- It is possible to observe dynamic changes in a cell by continuous scanning in the same plane.
- It can be used for visual analysis of expression patterns of genes during development.

Drawbacks and possible solutions

- It is relatively **slow t**o generate an image as it scans excitation spot point by point. It is not suitable to visualise fast processes.
- It is **not very sensitive** because PMT detectors record only a small percentage of the light that hit them.
- These limitations have been addressed by incorporating multiple pinholes and CCD camera as detector. In another variation- the multi photon excitation approach for optical sectioning eliminates the need for even a pinhole.

[You can listen to a video lecture on confocal microscopy by Kurt Thorn at ibioseminars.org]

SAQ 6

Which structure in a confocal microscope is used to obtain images from a single focal plane of the specimen sample?

2.8 SUMMARY

In this unit you have learnt that

- The cells of most organisms are smaller that what the human eyes is capable of seeing. A microscope is therefore an indispefisable tool because of the inherent limitation of the human eye.

- Most present day microscopes are compound microscopes that have two type of magnifying lenses.
- In order to generate good quality magnified image three most important parameter are magnification, resolution and contrast.
- Cells and tissues are generally fixed by physical or chemical methods to view them under microscopy.
- A bright-field microscope forms a dark image against a brighter background. In this the condenses concertrate and transmits the light directly through the specimen. In dark field microscopy, image forms against a dark background. It prevent direct light from entering the front of objective lense so microscope detect light scattered from the object. Most bright field microscope can be used for dark field microscopy by simply shifting the knob from bright to dark field.
- Phase contrast microscopy (PCM) and differential interference contrast microscopy (DIC) are contrast enhacing techniques. They were developed by Fist Zernike and G. Namarski respectively.
- Fluorescence microscopy is a special type of optical microscopy where a fluorescent sample is illuminated with light of shorter wavelength light usually blue or UV light and the emitted photons (longer wavelength) are measured. It find application in cell imaging immunofluorescence technique etc.
- Confocal microscopy includes a family of techniques that essentially have two parts-optical sectioning and confocal. It was developed by Marvin Minsky. Confocal microscopy produces high resolution in focus (confocal image). The image is created by scanning laser point by point over the fluorescent sample.

2.9 TERMINAL QUESTIONS

1. What is the difference between a simple and a compound microscope?
2. What are the important parameters for a good quality magnified image?
3. Indicate two ways by which the resolution of a microscope can be improved.
4. What is the difference between magnification and resolution?
5. Enlist the magnification and numerical aperture for each objective in your microscope. Can you deduce any correlation from the data?
6. What is fixation? What are the two general types of fixation?
7. What are the three types of filters used in fluorescence microscopy? Indicate their role.

8. What is immunofluorescence? What is the difference between direct and indirect immunofluorescence? Why is indirect immunofluorescence preferred over direct immunofluorescence?

9. How does a confocal microscope eliminate out of focus light?

2.10 ANSWERS

Self-Assessment Questions

1. a) A magnified image in a compound microscope is generated by a combination of twolenses: the objective and the eye piece (ocular). Refer to Fig. 2.2.

   b) The **Resolving power (d)** of a microscope is directly proportional to the wavelength of light used and inversely proportional to the numerical aperture of the microscope.

$$\textbf{Resolving power (d)} = \frac{\mathbf{0.612\,\lambda}}{\mathbf{n \sin \alpha}}$$

   (i) d = 0.61 × 400 / 1.4 = 174 nm

   (ii) d = 0.61 × 500/1.4 = 217 nm

   c) Magnification is 400X.

2. a) The advantage of fixing cells is that the samples can be preserved for a long time. Cell fixation prevents autolysis and decay of the specimen. Further, fixing also permeabilises the sample and makes it easier for dyes or antibodies against specific structures or proteins to gain entry into the cells.

   b) 1- d;2 - c ;3 – b; 4 - e ; 5 – a

3. a) **A bright field microscope** forms a dark image against a brighter background. In this the condenser concentrates and transmits the light directly through the specimen. A less transparent part of the sample would therefore appear dark against the bright background while more transparent parts will appear less dark and completely transparent part will have the same brightness as the background. In **dark field microscopy** the object is brightly illuminated against a dark background. It prevents direct light from entering the front of the objective and so the microscope detects light scattered from the object. The diffracted light will enter the objective and reach the eye. The condenser is equipped with a dark field stop to block the light.

   b) (i) False; (ii)False; (iii) True; (iv) True; (v) True

4.

Phase contrast microscopy (PCM)	DIC microscopy
It is not sensitive to sample orientation.	The contrast is directional and so sample orientation can change the regions of max and min contrast.
In PCM light traveling through the transparent sample is made to interfere with the illuminating light to produce contrast.	It is a variation of polarization microscopy. The condenser DIC prism splits illumination into two divergent polarised beams.
Thick samples can't be imaged properly.	Even thick samples can be imaged.

Please refer to section 2.5 for details.

5.. The shift in the wavelength of emitted light by an excited fluorochrome is called Stokes shift. However, fluorescence always occurs at a longer wavelength than the excitation light. The emission maxima of EGFP are 507nm which falls in the "Green" region.

6. This structure is the pinhole screen placed in front of the eye-piece.

Terminal Questions

1. A simple microscope has a single lens while a compound microscope has two sets of lenses. Refer to Box 1.
2. The resolving power, magnification and Contrast are most important parameters for getting a good quality image in light microscopy. Refer to section 2.2 for details.
3. The resolution of amicroscope can be improved by either using the shorter wavelength of light or by increasing the numerical aperture of objective lens.
4. The magnification power of a microscope is its ability to produce an enlarged or magnified view of an object to the eye.

   The ability of a microscope to allow distinction between two close but separate points in the specimen is defined as its resolution.

5.

Objective lens	Numerical aperture (NA)
4X	0.20
10X	0.45
40X	0.95
100X	1.4 with oil

The objective lenses with higher a magnification also has a higher numerical aperture. A higher numerical aperture means that even the oblique rays of light from the specimen will reach the lens and so the resolution will be higher.

6. Fixation is the process of preserving of cells / tissues in a near life like condition. It also protects the sample during further processing steps. Fixation can be done by either physical (temperature variations) or chemical methods (organic solvents or cross linking agents).
7. Three categories of filters are essentially required. They are excitation, barrier (emission) and dichromatic beam splitters (dichroic mirrors). The filters are high resolution with interference optics.
   a) The excitation filter transmits only the excitation light of the desired wavelength.
   b) Dichroic mirrors are placed in the light path at an angle and they reflect the excitation light from the objective to the sample.
   c) The barrier filter removes any excitation light that could otherwise reduce image's contrast. Filters enable the florescence light to form higher resolution of the final image.
8. Please refer to the last part of section 2.6 for applications of fluorescence microscopy.
9. By using a pinhole screen that allows only light from the focal plane to pass through and reach the detector.

2.11 SUGGESTED READINGS

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UNIT 3

ELECTRON MICROSCOPY

Structure

3.1 Introduction
Expected Learning Outcomes

3.2 Preparation of Biological Specimen
Fixation
Tissue Processing
Sectioning
Staining

3.3 Electron microscopy
Transmission Electron Microscope (TEM)
Scanning Electron Microscope (SEM)

3.4 Summary

3.5 Terminal Questions

3.6 Answers

3.7 Suggested Readings

3.1 INTRODUCTION

In Unit 2, you have learned about the principle, instrumentation and applications of various types of light microscopy. It is an indispensable tool for studying any biological sample at cellular and molecular level. The limited resolution and magnification power of light (optical) microscopy (LM) has led to the development of electron microscopy that has tremendously enhanced our understanding of the cell ultra-structure and function.

In electron microscopy, electrons are used to image the sample that has a higher resolution and magnification power than that of light. An electron has a much lower wavelength than visible light and so its resolving power is more. More than 85 years has elapsed since the first electron microscope was developed and technological innovations continue to provide higher resolution and variations to suit different needs.

Therefore, in this unit, you shall learn about the electron microscopy. This unit begins with the preparation of biological specimen and thereafter, you will learn about some key developments, instrumentation and applications of transmission electron microscope (TEM) and scanning electron microscope (SEM).

Expected Learning Outcomes

After completing the study of this unit, you should be able to:

- know the purpose and steps for specimen preparation;
- understand the significance of the fixation methods, sectioning and staining in specimen preparation;
- indicate the landmark discoveries and basic features of an electron microscope;
- explain why electrons have high resolving power than visible light;
- understand how electrons generate magnified image of specimen;
- describe the basic parts, working principle and application of the (TEM) and compare it with light microscope (LM);
- highlight the advantage(s) and disadvantages of TEM; and
- explain the working principle and applications of SEM.

3.2 PREPARATION OF BIOLOGICAL SPECIMEN

When we are observing small molecules or viruses they can be examined directly in the electron microscope, without elaborate preparation. All that is generally done is to use negative staining or some alternative method so that details are more clearly visible. On the other hand, most biological specimens require elaborate procedures before cells or tissues are ready for examination under an electron microscope.

The two major reasons for pretreatment are the **thickness** of the sample and its **water content** (70%). An electron microscope works at high vacuum and if untreated living cells are placed in it they would be dehydrated and lose their intricate structure. Apart from the high water content cells are generally, at least a thousand times thicker than a typical virus particle and so most electrons passing through it will be scattered and the resultant image will be uniformly dark. Therefore, cells are dehydrated and sectioned prior to observation.

In 1945, Porter, Claude, and Fullam used the fixation and staining methods to examine cells in tissue culture under the electron microscope. The basic steps include fixation, tissue processing, sectioning and staining. The Fig. 3.4 schematically depicts the steps of preparation of a biological specimen for electron microscopy. Let us proceed to learn them one by one.

3.2.1 Fixation

Fixation of tissue is the first and crucial step for specimen preparation. Biological specimen can be fixed either with chemical fixatives or freezing method. The advantages of tissue fixation are many. We have discussed this step in the preceding unit also as it is utilised in some forms of light microscopic observations. It serves to:

- preserve cells and tissue components as close to their living state as possible with minimum alterations,
- protect cell from autolysis and decomposition, and
- protect and stabilize cellular structure from subsequent treatments such as dehydration, staining and from irradiation by electron beam.

We will now describe the two common ways in use for the fixation of tissues or cells.

Chemical fixation method

Chemical fixation is a conventional method for preservation of biological specimen for microscopic studies. Tissue samples need to be dissected and cut into small pieces, approx. 1-2 cubic mm and quickly immersed in the fixative solution. However, chemical fixatives should be used in combination with buffering agents so that pH and osmolarity of the specimen is maintained. The duration of chemical fixation is generally two hours at room temperature. Microwaves can also be used to enhance the speed of fixation.

Chemical fixatives form cross links with biomolecules and provide long term stability and electron contrast. It is important to reduce artefacts that can arise during treatment. The two most common fixatives that are used in EM studies are **Glutaraldehyde and osmium tetroxide**. Glutaraldehyde is a primary fixative followed by osmium tetroxide (OsO4) as a secondary fixative. Both of them belong to the class of non-coagulant fixing agents.

In 1963 Sabatini, Bensch, and Barnett introduced glutaraldehyde as a tissue fixative for the electron microscopy. Glutaraldehyde (Fig 3.1a) is a five carbon dialdehyde fixative. The presence of the aldehyde groups makes it an efficient cross linking agent. It forms covalent cross links with lysine and arginine residues of cellular proteins (Fig. 3.1b) that helps to preserve the cellular architecture of the cell / tissue. Glutaraldehyde easily penetrates into small tissue and acts rapidly. It also denatures proteins that prevent tissue decay due to inhibition of enzyme activity. It has become the standard for electron microscopy. Recently glutaraldehyde has been identified as a powerful allergen and so safe concentrations are only recommended for use.

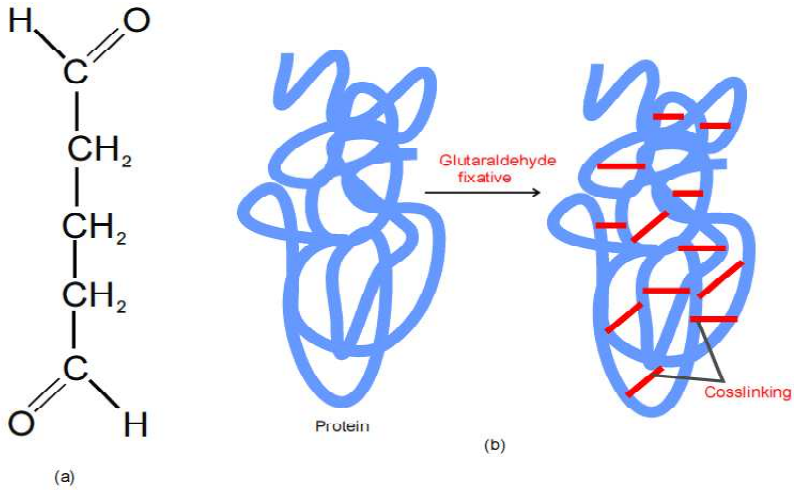


Fig. 3.1: (a) Structure of glutaraldehyde (b) Cross-linkage between glutaraldehyde and cellular proteins.

Osmium tetroxide: Osmium tetroxide (OsO_4) is used as a **secondary fixative** after glutaraldehyde fixation. The structure of OsO_4 comprises of four double bonded oxygen atoms attached to the osmium atom (Fig. 3.2). However, its penetration rate is slower than glutaraldehyde. It fixes by forming cross links with unsaturated lipids (and probably proteins) and it also enhances contrast by adding electron dense material to the section. It is primarily used to stabilize the membrane structures of cells particularly fatty tissues and membrane lipids.

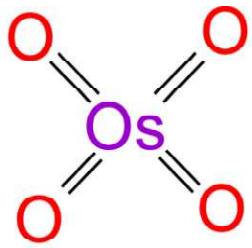


Fig. 3.2: Structure of osmium tetroxide (OsO_4)

The constituents of the cell that do not react with the fixatives are not necessarily preserved and may even be washed out in the subsequent processing steps. It is largely the macromolecular skeleton of the cell that is most likely to be preserved.

Freezing method (Cryofixation)

The freezing of biological samples is another way to halt cellular processes rapidly, especially for cells or tissues that cannot be fixed with chemicals. In 1957, Steere initially developed the Freeze-fracture technique (to split open samples) which was improved later by Moor and Muhlethaler.

Fixation of tissues or cells (specimen) using the freezing method is known as **cryofixation**. It is technically challenging and requires expensive equipment. In this method, the specimen is frozen by a process of **rapid cooling,** the rate of which will be fastest at the edge of the sample. The basic aim of Cryofixation is to VITRIFY the water in the sample and the state of water changes from liquid to solid state without intermediate ice crystal formation. As a result, the structure of specimen is preserved close to its native state.

There are several freezing techniques currently available that provide rapid cooling of the sample such as high pressure freezing that exposes the sample to high pressure (approx. 2000 bar) prior to freezing or by using liquid nitrogen or liquid helium. An entire new field called cryo electron microscopy has branched from this technique.

The main advantage of freezing technique is that frozen tissue can be cut directly with a special microtome (cryo-microtome) as freezing makes the

tissue solid enough to allow ultrathin sectioning. If there is a cryo stage the frozen samples can be imaged directly. Cryofixation is generally used to view cross sectional cell surface and for immunohistochemical studies of cells.

3.2.2 Tissue processing

Tissue processing is next step after chemical fixation of tissues that involves **dehydration** followed by embedding process. During tissue processing, water molecules are removed from the specimen by freeze drying or dehydration in alcohol or acetone. The dehydrated tissue is immersed in a solution of unpolymerised plastic embedded resin such as Epon or Araldite epoxy resin and acrylic. In 1956, Glauert and colleagues used epoxy resin Araldite for the first time as an embedding agent for electron microscopy.

When unpolymerised solution has penetrated the specimen completely then the plastic is polymerized usually by application of heat. Both these materials are strong materials yet soft enough to permit sectioning. They also cause minimal distortion of the sample as they polymerise without much change in volume. **Embedding** makes the tissue hard and provides mechanical support for ultra thin sectioning. The specimen may require polishing and this is done using ultra-thin abrasives.

3.2.3 Sectioning (cutting)

After embedding, ultrathin sectioning of tissue / cells must be performed delicately. The sectioning of specimen with precision is done with **ultra-microtome** (Fig.3.3) which was developed by Porter and Blum in 1953. It is a slicer in which the knife is kept stationary and the specimen is moved at each stroke. The knife is made of glass, diamond or sapphire. The glass knifes can be made in the lab and are therefore cheaper. The optimum size of ultrathin section is about 0.5 µm or less in EM. The sections are fragile and so they are cut directly into a trough mounted on the back of the blade. The trough is filled with water or 10-20% ethanol in which they “float out” and then picked on grids for support in the microscope. The whole process is observed under the microscope.

Fig. 3.3: Ultramicrotome (Courtesy: Jiwaji University, Gwalior, India)

3.3.3 Staining

Staining is the last step in which the sectioned tissue is stained with a solution of heavy metal salts to increase the light scattering power of the section. A number of stains are available to enhance contrast as many biological materials are transparent to electrons. These stains fall into two major categories - positive and negative stains. A drop of heavy metal salts is deposited onto to sectioned tissue and then the stained specimen is placed on a small circular metal grid for viewing the image under the electron microscope. The sample can also be stored and observed later.

In positive staining, the image appears as dark area against light background as these stains deposit electron dense material on the area of interest. Lead citrate and uranyl acetate are common stains. The positive stains are used to view specific molecules within cells, for instance proteins, lipids and DNA. The Uranyl ions react strongly with phosphate and amino groups, staining DNA and some proteins whereas lead citrate is taken up more strongly by lipid containing membranes and it produces a general increase in contrast.

The negative stains are used to view intact biological structure such as sub cellular organelles, bacteria, viruses. These stains penetrate and darken the interstices between areas of interest and produce an image in which the specimen appears light against a stained dark background. Negative staining technique developed by Brenner and Horne 1959.

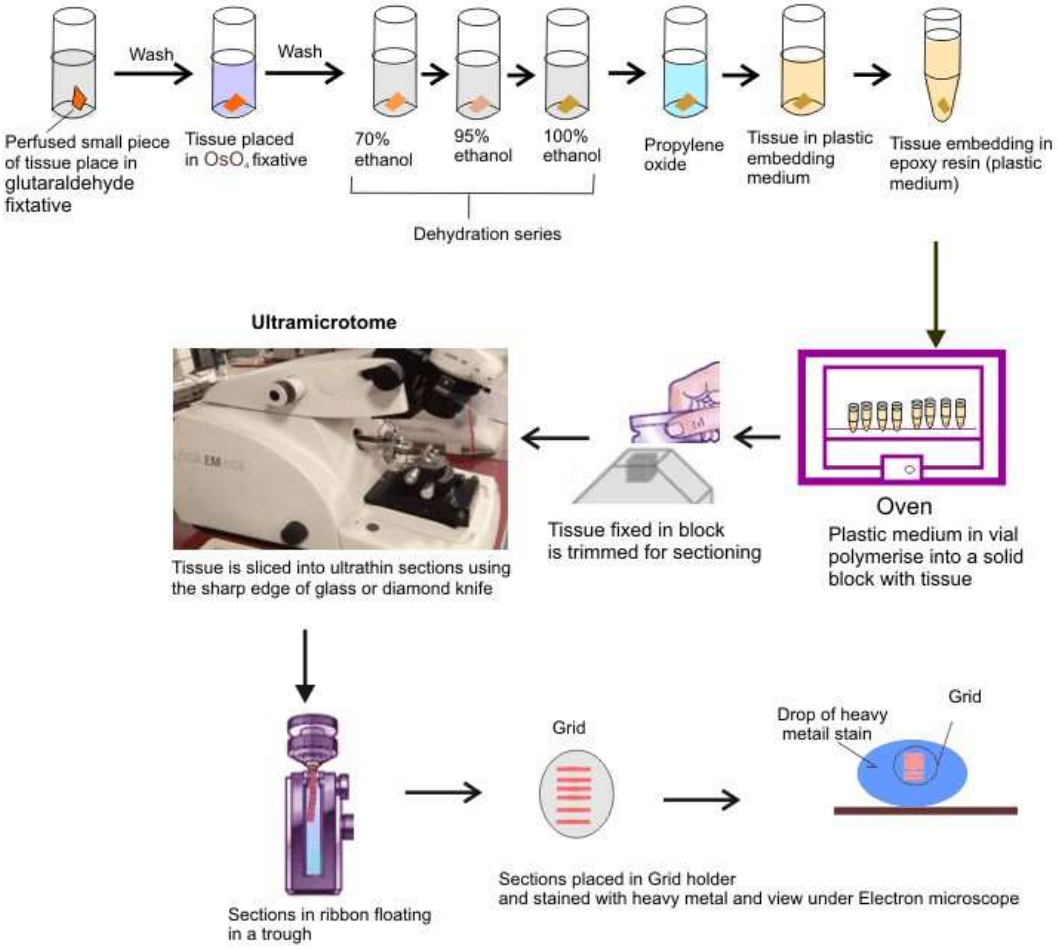


Fig. 3.4: The steps of sample preparation for Electron microscopy

SAQ 1

a. Fill in the following blanks with correct answer:

i) Glutaraldehyde reacts with …….and osmium tetroxide reacts with ……during tissue fixation

ii) A technique for fixation of tissues or cells by freezing is called.....

iii) Sectioned tissue is stained with ……..

iv) In EM, optimum thickness of sectioned specimen is……..

b) Why specimen fixation is necessary for electron microscopy?

3.3 ELECTRON MICROSCOPY (EM)

The theoretical resolution of an optical light microscope is 200 nm. This barrier to higher resolution had to be overcome for detailed ultra-structural investigations to be possible. The first prototype of the transmission electron microscope built by **Max Knoll** and **Ernst Ruska** in 1931 finally showed the way. Two years later Ruska made an electron microscope (Fig. 3.5) that exceeded the resolution of an optical microscope. The earliest electron microscopes only proved the basic idea of using electrons beams as an alternate source of illumination could generate a higher resolution image than the best light microscopes. More than 85 years has elapsed since the first electron microscope was developed and technological innovations continue to provide higher and higher resolution and variations to suit different needs. The improved resolution has been a benchmark for defining the progress of technology.

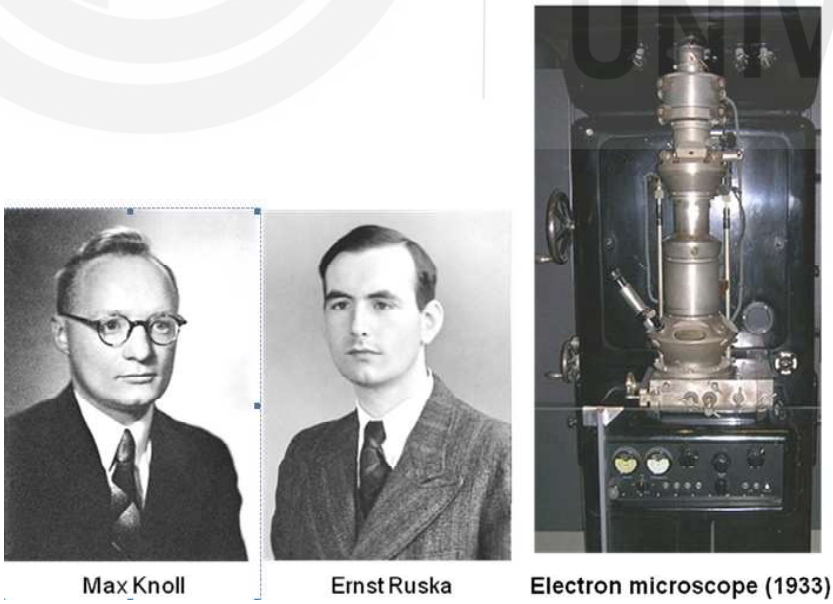


Fig 3.5: A view of the earliest transmission electron microscope

Once the potential of the electron microscope was recognised advancements were made in multiple directions with the aim of ultimately achieving atomic resolution. The improvements were made in electron lens to minimize aberrations; increase in accelerating voltage of the electron beam;

J.J. Thompson in 1897 discovered the electron. At that time, they were considered as a particle.

Later, **Louis DE-Broglie**in 1924 proposed an idea on wave properties of electrons which was subsequently confirmed experimentally.

Today electrons are better described as having a dual character.

development of better vacuum systems and brighter electron sources such as lanthanum borohydride and field emission electron guns used for producing high energy electrons. In addition, the possibility of tilting specimen stages has facilitated the examination of the specimen from different angles it has proved very useful in the determination of crystal structure.

In the long history of inventions and innovations in electron microscopy some of the key landmarks that needs special mention is the development of **electromagnetic lenses** by Hanse Busch in 1931; the use of electron microscope by E. Ruska in 1937 to view biological specimens and the invention of the scanning electron microscope (SEM) in the same year by Manfred von Ardenne. In the following year Ruska at Siemens produced the first commercial Transmission electron microscope (TEM). The first commercial SEM was introduced only in 1965. The present-day electron microscopes can no doubt (magnify) more than two but million times, they are still based on Ruska's prototype.

The late 1980s and throughout the 1990s are notable for the development of the so called '**environmental electron microscopes'** that allow investigators to examine samples under more natural conditions of temperature and pressure. This has tremendously expanded the types of samples that can be examined.

Before we proceed to understand the instrumentation and applications of electron microscopy it is worth mentioning that the acceptance of the **wave nature of lectrons** was instrumental in the use of electron beams as the source of illumination for improving the resolution of the microscope.

The imaging power of electron microscopes depends on the electron wavelength. An electron has shorter wavelength **(λ =0.004 nm**) than visible light (λ =400 nm). As you know the limit of resolving power is directly proportional to the wavelength of illumination *i.e*. shorter the wavelength, higher is the resolution and vice versa.

The wavelength of an electron varies with the speed at which the particle is moving in vacuum, which in turn depends on the accelerating voltage applied. An approximate relationship that relates the wavelength of an electron with the accelerating voltage is:

$$\lambda = \sqrt{150/V}$$

Where is the wavelength of electrons in angstroms (Å) and *V* is the accelerating voltage in volts (V).

Standard TEMs operate at a voltage range from 10,000 to 100,000 V. At 60,000 V, the wavelength of an electron is approximately 0.05 Å. The effective wavelength of an electron is about 10,000 times less than that of light wave. This single factor would lead to a considerable increase in the resolving power. The magnification achieved ranges from about 10,000 – 250,000X.

Now let us look at a modern electron microscope **(Fig. 3.6)** Basically an Electron microscope comprises of a tall hollow cylindrical vacuum column through which the electron beam passes and a console containing a panel of dials that electronically control the operation in the column. The top of the

column contains the cathode, a tungsten wire filament that is heated to provide a source of electrons about which you will study in next subsection. The whole process for specimen examination in the EM is performed under the vacuum (without air).

The next step is to understand how electrons (e^-) interact with the matter and how electrons generate an image of the sample. Recall the basic properties of electrons from your chemistry course at 10+2 level.

Fig 3.6: Electron microscope
(Courtesy: Jiwaji University, Gwalior, India)

In TEM, a beam of electrons has very high energy of the order of 100-10000 KV. This simply means they move at high speed as they go through matter. You can see in Fig.3.7 that some electrons from an incident electron beam pass through matter and some electrons simply don't interact with an atom and they are the **unscattered** electrons. Some of them interact with the nucleus of an atom and their direction is changed. This process is called **inelastic scattering** in which electrons neither loses energy nor changes speed but they change direction. Alternatively, some electrons are either absorbed or transmitted by the sample. As a result, they lose energy which is known as **elastically scattered electrons**. These electrons are very significant to generate an image of sample in TEM.

Besides, some electrons interact only with the electrons of an atom of the sample and they either **backscattered** or generate **secondary electrons** which are responsible to imaging of surface of sample in SEM.

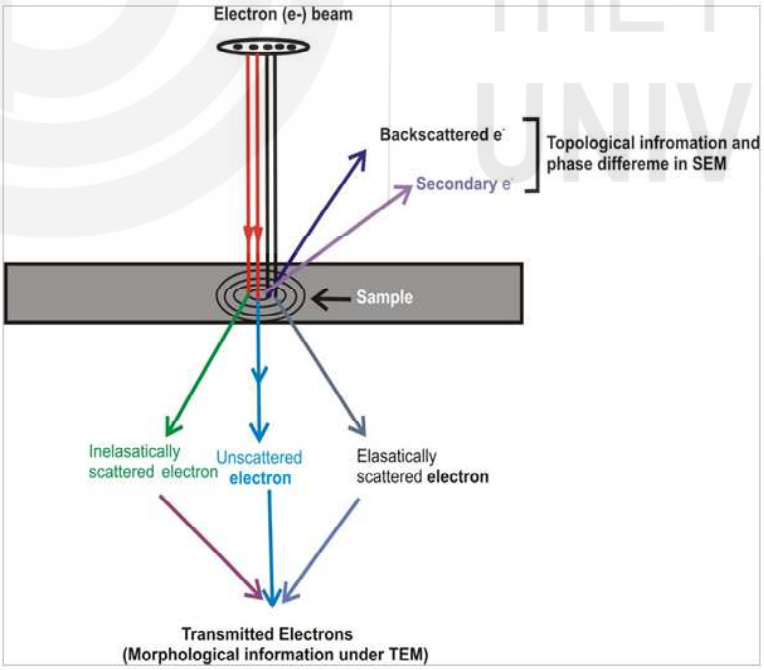


Fig. 3.7 : Interaction of high energy electrons with matter in Electron microscopy

In modern science, the electron microscopy is a key research tool in the field of histochemistry and medical sciences to examine biological materials for diagnosis of diseases and biopsy samples. It is also used to study surface characteristics of cells, ultra-structure of cells, protein localisation, 3D tissue imaging and toxicology. Today there are two major types of electron

microscopes used in clinical and biomedical research namely, Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM).

3.3.1 Transmission Electron Microscopy (TEM)

In TEM, electrons pass through the specimen to form the image of internal structure of the specimen. It requires ultra thin sections for electrons to penetrate with ease. The specimen is placed at the position along the direction of the electron beam.

The source of illumination in an electron microscope is the **electron gun** that is fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by the anode at very high voltage. The effect of the gun is to produce a narrow beam of electrons passing at high speed in a given direction. They are focused on the specimen by the electromagnetic lenses. The electrons that transmitted through the specimen are either unscattered or scattered electrons depending on their interaction. The emerging beam carries information about the structure of the specimen and it is further magnified by other lenses to generate a high resolution image at atomic level. The image is recorded by projecting it on a fluorescent screen or photographed using charged coupled device (CCD) camera.

The basic instrumentation of the TEM comprises of an electron emission source, electromagnetic lenses, fluorescent screen and image recording system which is summarized in the Table 3.1 along with their role (s).

Table 3.1: Basic parts of TEM

Parts of TEM	Role(s)
1) A large cylindrical vacuum column	Vacuum state is required in order to prevent the collision of moving electron beam with gas molecules.
2) An electron gun (voltage 5-100 KV)	Source of electron beam
3) Electromagnetic condenser lens	collect and focus the electron beam onto the biological specimen
4) Electromagnetic objective and project or lenses	They collect and receive the transmitted electrons from the specimen and produce a magnified image on viewing screen.
5) Fluorescent screen	Collecting and analyzing the image
6) The image-recording system	Image transmitted to the digital camera for recording.

A liver cell (hepatocyte) shown in Fig.3.8 is imaged both with a light microscope (400X) and transmission electron microscope (6200 X) to give an idea of the resolving power of an electron microscope. The light microscopic image shows the general cellular morphology while TEM image reveals the presence of sub cellular organelles including mitochondria, endoplasmic reticulum and nucleus surrounded with the nuclear membrane.

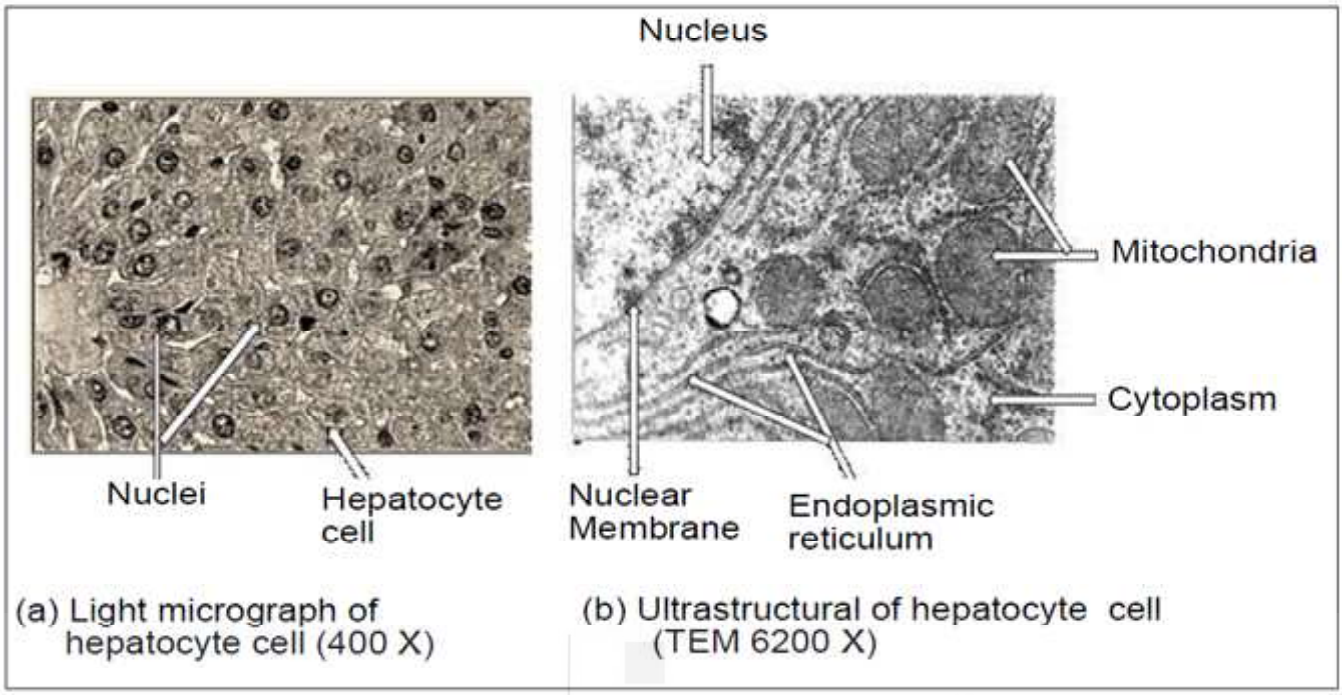
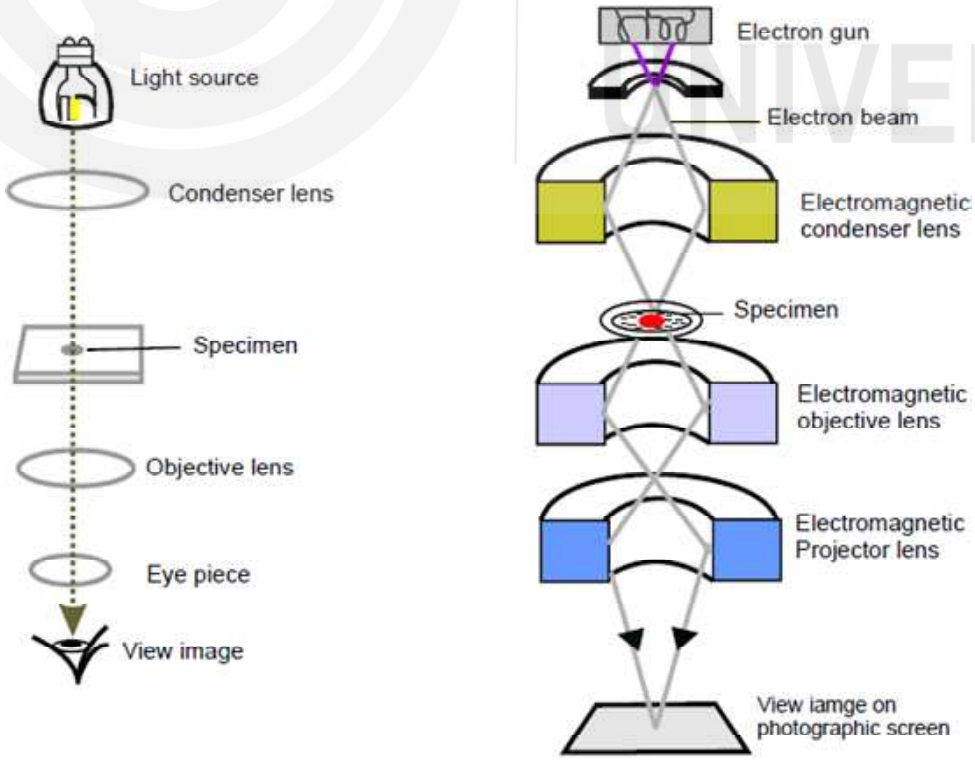


Fig. 3.8: A comparison of a hepatocyte cell image seen under a light microscope and a transmission electron microscope.

TEM is widely used in Cell and Molecular biology research for the histological analysis of cells and tissue, drug investigation, diagnosis of diseases and the study of sub cellular organelles such as nucleus, mitochondria, endoplasmic reticulum etc. and biomolecules.

Now you can attempt to **compare** a light microscope and a transmission electron microscope by carefully observing Fig. 3.9.



(a) **(b)**

Fig. 3.9: Basic parts of (a) an optical microscope and (b) A Transmission electron microscope (Source: The Cell: A Molecular Aporoach, 2009)

In general the layout of an EM is basically similar to a light microscope except that it is inverted. The source of **illumination** is an electron gun in an EM in place of a lamp. The image formation in a LM depends on the degree of light absorption whereas in a TEM it depends on electron scattering.

The **lenses** of LMs are glass lenses and focusing is done by moving them nearer or farther away from the specimen. In TEMs all the lenses are electrostatic and electromagnetic coils and focusing is done by manipulating the amount of current flowing through the magnetic coils.

The equivalent of an **eye piece** of a LM is the **projector lens** in an EM as it projects the final image. The other lenses such as the condenser and objective perform similar functions in both. The final image in a LM is viewed by the eye whereas in a TEM it is formed on a viewing screen or photographed.

An EM has the major advantage of increased resolution over a LM. The resolution of TEM is limited primarily by spherical aberration. Some electron microscopes can magnify specimens up to 2 million times, while the best light microscopes are limited to magnifications of 2000 times.

TEM also has some **disadvantages**. They are (i) Live biological material cannot be observed since electron beams traverse in vacuum, (ii) Only very small area of tissues can be viewed at a time, (iii) Biological samples need elaborate **processing and sectioning** before they can be observed under EM, (iv) The operation of an EM requires maintenance of extremely stable voltage supply, ultra high vacuum systems and a continuous cooling system through the lenses and pumps. Therefore, a LM and a TM are complementary to each other.

Electron microscopes are **expensive** as compared to most commonly used light microscopes and high resolution versions can be handled only by trained technicians. Some of these limitations have been taken care of by different manufacturers by making low resolution cheaper versions suitable for most needs and many of them do not even require rigorous training.

3.3.2 Scanning Electron Microscopy

The scanning electron microscope (SEM) is another variation of an electron microscope. The basic parts of SEM are similar to the TEM except it has a **secondary electrons detector**. SEM produces an image by detecting low energy **secondary electrons** that are reflected back from the surface of the specimen due to excitation by the primary electron beam or backscattered electron signal. These secondary electrons strike a detector that is located near the surface of the specimen as shown in Fig. 3.10. SEM uses a fine beam of accelerated electrons that scans line by line on the specimen surface to deliver an image with information of surface topology. The detectors build up the image by mapping the detected signals with beam position. Therefore, the Image formation in the SEM is indirect. The resolution limit of SEM is about 3-15 nm as compared to resolution of 0.2 nm by TEM but SEM has a greater depth of view and the images produced closely represent the 3D structure of the sample.

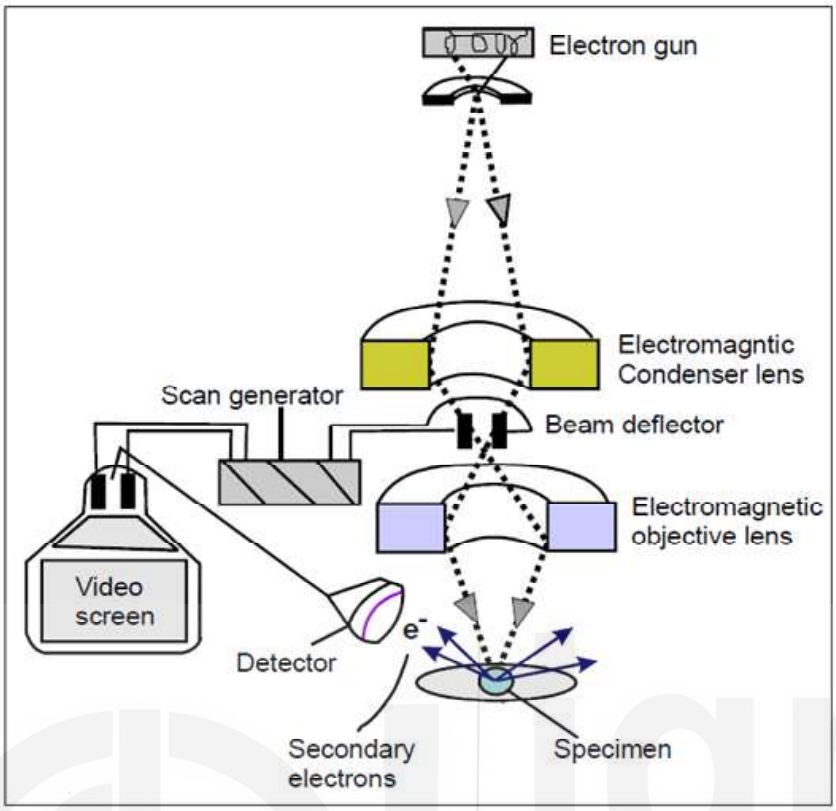


Fig. 3.10 : Basic setup of Scanning electron microscope (SEM)

(Source: Essential Cell Biology, Garland Science)

The SEM is primarily used to study of topography, composition and morphology of surface of the biological specimens. Standard SEM also provides three-dimensional image of cell surface. The **Fig. 3.11** shows the cell surface image of the human red blood cells (RBC) obtained by the SEM electron microscopy. The RBC has a biconvex shape.

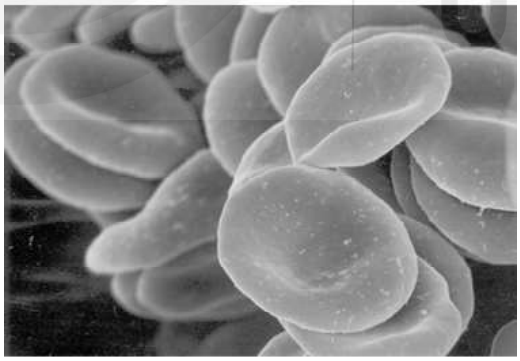


Fig. 3.11 : Cell surface image of RBC (Credit: Tina Carvalho, 2011) CIL:221, Homo sapiens, erythrocyte. CIL. Dataset.**)** (https://doi.org/doi:10.7295/W9CIL221)

SAQ 2

a) Which microscope will you use in the following cases? Answer in the space provided below.

i) To examine mitochondria within the cell. …….

ii) To obtain the cell surface image of RBC cells……..

iii) To view cell morphology……..

b) Fill in the following blanks with correct answers:

i) The TEM uses ……..electrons and the resultant image is projected on a ……screen.

ii) In SEM ……are used to generate ……..image of the cell surface.

iii) The resolution limit of the TEM is ……..nm while it ……..nm in case of SEM.

3.4 SUMMARY

- Electron microscopy is a key laboratory tool to investigate and analyze the biological sample at atomic level due its higher resolution and greater magnification power over the LM.
- Biological specimen must undergo special treatment prior to examination by electron microscopy. The specimen is initially fixed by using either Chemical (Glutaraldehyde and Osmium tetroxide) or physical (cryofixation) methods followed by dehydration, sectioning and finally staining.
- The imaging power of electron microscopes depends on the electron wavelength. An electron has shorter wavelength **(λ =0.004 nm**) as compared with visible light (λ =400 nm) and hence EM achieves higher resolution. In TEM, the electron beam is transmitted thought the sample and focused on a photographic screen to generate a magnified image. In SEM, secondary electrons are used to get topological information view the surface image of the specimen.
- Both TEM and SEM are widely used to study of tissue, cells and biomolecules. It is also used in toxicological studies and biomedical investigations. TEM is used to study ultrastructural details of cells and SEM is useful to analyze the three-dimensional surface details of biological specimen ranging from human cells to viruses.

3.5 TERMINAL QUESTIONS

1. Differentiate between the light microscopy and electron microscopy.
2. Define the following steps of specimen preparation:
   i) Chemical fixation
   ii) Dehydration
   iii) Staining
   iv) Sectioning
3. What is cryofixation?
4. Explain how electrons produce a high resolution image?
5. Give the main difference in the working principle of TEM and SEM.
6. What are advantage(s) and disadvantage(s) of EM?

3.6 ANSWER

Self-Assessment Questions

1) a) i) proteins and forms cross links; unsaturated lipids
   ii) Cryopreservation
   iii) Heavy metal salts
   iv) 0.5µm or less thick.

   b) Fixation is crucial step in specimen preparation. Samples can be fixed by either chemical or physical method. Fixation protects the specimen (tissue or cells) from the autolysis and decomposition and provides stability to the cellular structure and preserves them with minimal alterations. Refer to section 3.2.1 for details.

2) a) i) Transmission Electron Microscopy (TEM)
   ii) Scanning Electron Microscopy (SEM)
   iii) Light (or optical) Microscope (LM)

   b) i) Primary transmitted electrons; fluorescent screen.
   ii) Secondary electrons; three-dimensional image.
   iii) 0.5 nm; 10-15nm

Terminal Questions

1. Differences between Light Microscope and electron microscopy

Characteristics	**Light Microscopy (LM)**	**Electron microscopy (EM)**
Light source	Visible light	electron beam
Lens	Optical glass Lens	Electromagnetic lens
Resolution and magnification	Lower resolution and magnification as compared to an EM	Higher resolution (100 µm -0.1 nm) and higher magnification power (about 200,000X) than a LM.
sample view	Directly viewed (Human eye)	Photographic film
Fixation	required	Required
Specific condition	Not required	Vacuum system
Sample staining	Moderately required	Staining with heavy metals

2. (i) **Chemical fixation** is a conventional method of preserving the tissue or cells by treating the sample with chemical agents. Glutaraldehyde and osmium tetroxide are generally used for tissue fixation for electron microscopic studies. They bind biomolecules and cross linkage them. Please refer to sub-section 3.2.1 for details.

(ii) **Dehydration** is the process of reducing / removing water from a specimen by ethanol treatment. Please refer to subsection 3.2.3.

(iv) **Staining:** The ultrathin sections are stained with heavy metal salts to enhance contrast of biological samples. The stains used may either be positive or negative stains. Please refer subsection 3.2.4

(iii) **Sectioning** is the cutting the sample into ultrathin sections with the help of an ultra microtome. Please refer to subsection 3.2.3.

3. **Cryofixation** is a freezing method in which the specimen is fixed by rapid cooling. The structure of the specimen is preserved close to its native state.

4. In electron microscopy, the image depends on how electrons interact with matter. There are four types of electronic interactions with matter. They are: (a) **Unscattered electrons** do not interact with matter as they pass through it. (b) **Inelastic scattering**: electrons change direction after interaction with matter. (c) **Elastically scattered** electrons loss energy after interaction and this type of electrons play an important role in producing an image in TEM. (d) Some electrons are **back scattered** or generate secondary electrons after interaction with atom of matter. These electrons are useful to scan and form a surface image of specimen under SEM.

5. In TEM, primary transmitted electron beam is used to examine the ultra structure of cells and in SEM, low energy secondary electrons (backscattered electrons) are used to form three-dimensional image of the cell surface that provide information of topology of specimens.

6. **Advantage:** TEM has higher resolution and able to magnify specimen image up to 2 million times over a LM (2000 times). It is routinely used to examine the ultra structural image of cells in the biomedical research.

   Disadvantages: In TEM, (i) the live biological material cannot be observed since electron beams traverse in vacuum,

   ii) Only very small area of tissues can be viewed at a time,

   iii) Biological samples need elaborate preparation before observation under EM,

   iii) The operation of an EM requires maintenance of extremely stable voltage supply, ultra-high vacuum systems and a continuous cooling system through the lenses and pumps.

3.7 SUGGESTED READINGS

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UNIT 4

CENTRIFUGATION

Structure

4.1 Introduction
Expected Learning Outcomes

4.2 Principle of Centrifugation

4.3 Instrumentation: The Centrifuge
Type of rotors
Type of centrifuges

4.4 Differential Centrifugation
Subcellular fractionation

4.5 Density Gradient Centrifugation
Rate-Zonal Centrifugation
Isopycnic Centrifugation

4.6 Safety measures and centrifuge maintenance

4.7 Summary

4.8 Terminal Questions

4.9 Answers

4.10 Suggested Readings

4.1 INTRODUCTION

So far in the preceding units of Block 1 you have learned about cells, their structural organisation in prokaryotes and eukaryotes and the role of various sub-cellular structures. In addition, the principles and applications of Optical and Electron microscopy were elaborately discussed , as microscopy is an important tool in the study of cells and their structural organisation.

An approach used by biologists to study structure and function of cell constituents is called the **reductionist approach**. It depends on the separation of cell constituents and then studying them in isolation. Biologists have used centrifugation as one of the tools to separate biomolecules macro molecular complexes and organisation. Therefore, centrifugation is a rountine laboratory technique in modern biochemical, cellular and molecular biological studies.

Centrifugation is a technique that uses centrifugal force to separate and purify biological components such as cells and subcellular organelles, suspended in liquid medium. The particles are separated at different sedimentation rate according to their size, shape or density in an applied centrifugal force.

This is the last unit of Block 1 deals with the principle, types and application of centrifugation. This unit begins with principle of centrifugation. Here you will learn about sedimentation coefficient, Svedberg unit and relative centrifugal force. This will be followed by a description of the type of centrifuges and rotors. You will also learn about the working principle and applications of differential centrifugation and density gradient centrifugation (Rate Zonal and

Isopycnic centrifugations) which are widely used in Cell and Molecular biology. The unit ends with safety measures and centrifuge maintenance. In Block 2 you will study about structure and functions of sub-cellular organelles.

Expected Learning Outcomes

After completing the study of this unit, you should be able to:

- explain the basic principle of centrifugation and Svedberg Unit;
- describe the following type for contrifuge and their applications:
  - Benchtop centrifuge
  - Micro centrifuge
  - Refrigerated centrifuges
  - Ultracentrifuge
- differentiate between the three type of rotors and their selective usage;
- explain the principle of differential centrifugation;
- indicate the use of differential centrifugation for subcellular fractionation;
- explain the working principle of the density gradient centrifugation (rate zonal centrifugation and density gradient centrifugation);
- know the relevance of density gradient media in density gradient centrifugation; and
- Understand the importance of practicing safety measures and centrifuge maintenance.

4.2 PRINCIPLE OF CENTRIFUGATION

The basic principle of centrifugation is based on sedimentation. Sedimentation is the tendency of particles in liquid suspension to settle down gradually to the bottom of the tube under the influence of the earth's gravitational force. When a particle moves in a circular motion, it experiences two forces *i.e*. centrifugal force which is outward force and centripetal force which is towards the central axis (Fig. 4.1).

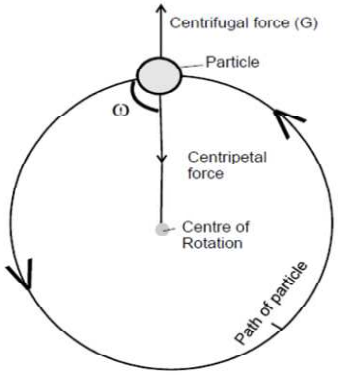


Fig. 4.1: Centrifugal and centripetal forces acting on a moving particle

Consider a particle of mass, m_o, suspended in a liquid of density, ρ, it would experience an up thrust in accordance with Archimedes's principle that is equivalent to the weight of displaced liquid. The buoyant mass (m) of the particle would now be minus the correction factor for the up thrust.

$$m = m_0 - m_0 v^{-}.p \qquad \text{Eqn (i)}$$

where, v^{-} is the partial specific volume of the particle.

Now if the particle referred above is subjected to a centrifugal field, it will experience a centrifugal force, G, that is dependent on the angular velocity (ω) of rotation (radians / sec. with one revolution = to 2 π radians) and the radial distance (r) of the particle from the rotation axis (cm) as shown in Fig. 4.1.

$$G = m.\ \omega^2 \cdot r \qquad \text{Eqn. (ii)}$$

Combining eqn (i) and (ii) directly relates mass, angular velocity and distance from the centre of rotation to the centrifugal force experienced by the particle.

$$G = m_0\ (1 - v^{-}.\ p)\ \omega^2 \cdot r \qquad \text{Eqn (iii)}$$

The movement of the particle through the medium is hindered due to frictional force (F), operating in opposite direction. It is given as:

$$F = f\ .v \qquad \text{Eqn (iv)}$$

where, f is the frictional coefficient. It is dependent on the shape and mass of the particle; viscosity of the solvent and the velocity (v) of the particle.

As centrifugation progresses, a constant velocity (sedimentation velocity) is reached. Then, G =F and,

$$m_0\ (1 - v^{-}.\ p)\ \omega^2 \cdot r = f\ .v$$

The sedimentation velocity, $v = m_0\ (1 - v^{-}.\ p)\ \omega^2.r / f$ Eqn. (v)

According to the Eqn.(v)

- Larger particles tend to move faster than smaller ones;
- A denser particle moves faster rate than less dense ones;
- The denser the solution, more slowly the particle will move, and
- Greater the frictional coefficient, the slower the particle will move.

It is generally difficult to determine v, ρ and f accurately so sedimentation coefficient (s) is a useful measure used to describe the differential behavior of particles in a centrifugal field.

$$s = \frac{\omega^2 r}{v} = \frac{m\ (1 - v^{-}\rho)}{f} \qquad \text{Eqn. (vi)}$$

The sedimentation coefficient (s) is the ratio of sedimentation velocity (v) and centrifugal force (G). It is a constant for a given particle or solvent. More often, the notation provided by **Theodor Svedberg** is used to describe sedimentation coefficients. One Svedberg is taken as 1×10^{-13} seconds. Many biological components are described by their sedimentation coefficients. The

Theodor Svedberg

more the S value the faster a particle will sediment, for instance ribosomes from prokaryotes sediment at 70S and their subunits at 50S and 30S. The S values of the ribosome subunits do not add to that of the intact ribosomes as the shape of the particles also influence S values, so the relation between molecular mass and sedimentation coefficient is not always simple.

It is often convenient to express centrifugal force as a value relative to the Earth's gravitational field as relative centrifugal force (RCF).

$$\mathbf{RCF = (1.12 \times 10^{-5}).\ (rpm)^2\ x\ r} \qquad \text{Eqn. (vii)}$$

Where 1.12×10^{-5} is an empirical factor,

r = radius of rotor from the center of rotation,

rpm= revolutions per minutes

Hence, RCF as defined in Eqn (vii), depends on the rpm and the radius of rotation and it is generally given in **units of gravity** or **× g**.

SAQ 1

Answer the following questions:

a) Explain the basic principle of centrifugation.

b) What is meant by Svedberg Unit?

4.3 INSTRUMENTATION: THE CENTRIFUGE

The centrifuge is a common laboratory instrument used for centrifugation. It is generally a closed compartment consisting of an electric motor, the rotors and a drive shaft (Fig. 4.2). The rotor is the main part of the centrifuge in which the sample tubes are placed for separation. Let us now proceed to study the rotor types and then centrifuges suitable for different needs.

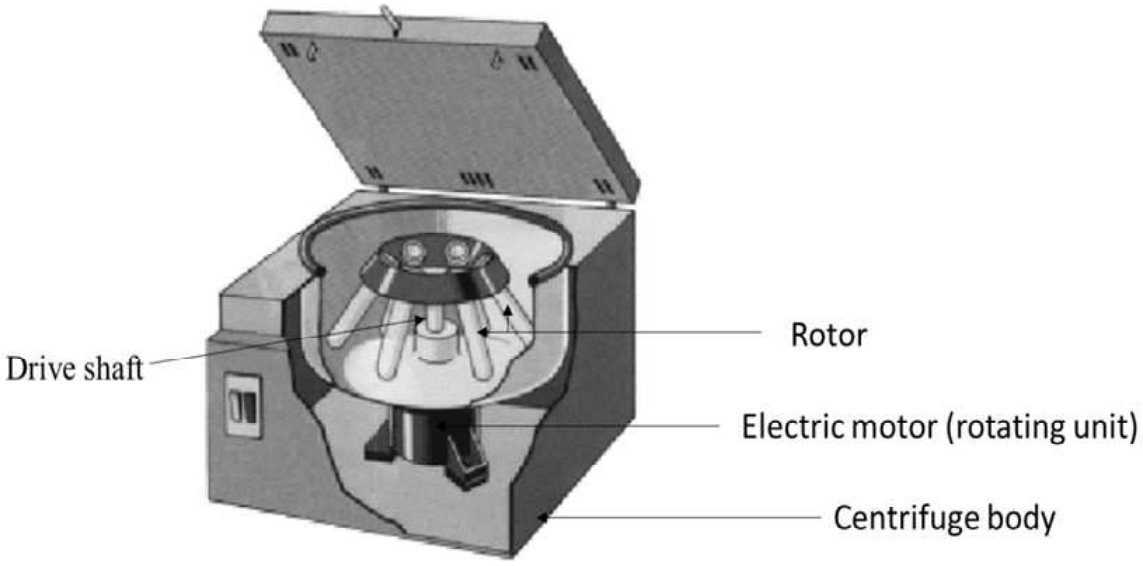


Fig. 4.2: The Centrifuge

4.3.1 ROTOR

Rotor is an important part of the centrifuge. It is the **rotating unit** that produces centrifugal force on particles to be separated. The rotor rotates around a spindle .A rotor is made up of either aluminium, titanium, fiber reinforced composites or metal alloys. Depending on the material they are made up of, they are suitable either for low or high speed centrifugation.

Rotors are generally described by their type; sample volume capacity and number of tubes and maximum speed. There are three type of centrifuge rotors based on the position of tubes relative to the axis of rotation. They are:

1. Fixed-angle rotor,
2. Swinging-bucket rotor, and
3. Vertical rotor

Fixed-angle rotor: In a fixed angle rotor the tubes containing the sample are placed in the openings in the rotor that are at a fixed angle relative to the axis of rotation (Fig. 4.3). The tubes remain in that position during the run, regardless of the rotor speed. As the rotor begins to rotate, the sample tube reorients to the axis of rotation and particles migrate towards the wall of the tube and sediment asymmetrically at the bottom of the tube. The sedimenting particles have to travel a short distance before pelleting. Fixed angle rotors can accommodate more tubes as compared to swinging bucket and they can also withstand much higher gravitational forces making it the most widely used rotor type. Fixed-angle rotor is useful for differential centrifugation and density gradient centrifugation.

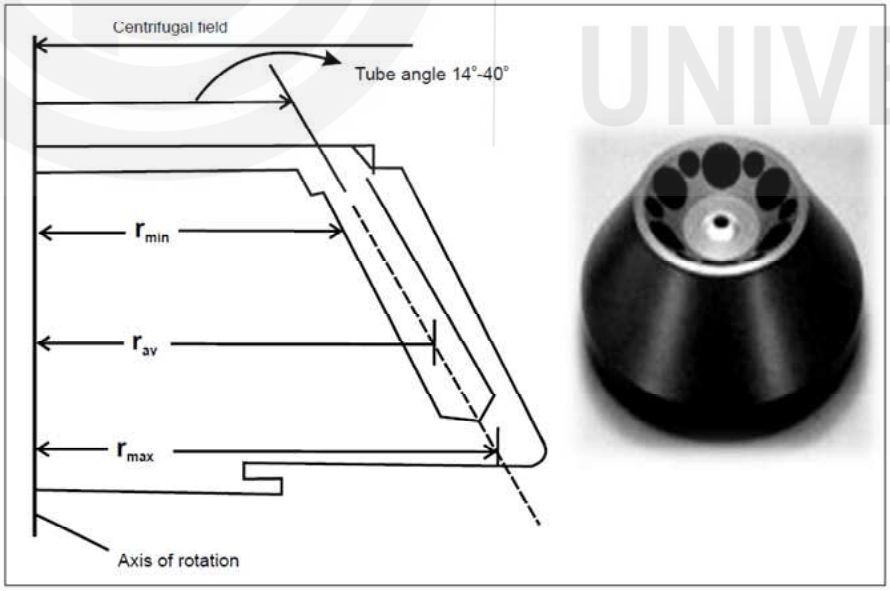


Fig. 4.3: Fixed angle rotor

Swinging-bucket rotor: A swinging bucket or horizontal rotor has hinge pins through which the sample containers (buckets) are attached to the rotor body. The tubes swings out from the vertical to a horizontal position when force is applied and the containers are at an angle of 90° angle relative to the angle of rotation and parallel to the applied centrifugal field (Fig 4.4). The bucket returns

back to vertical position as the centrifuge decelerates. The pelleted material is symmetrically distributed at the bottom of the tube. It is easier to drain supernatant without disturbing the pellet. This is an ideal rotor for separation of large volume samples (up to 12 L) at low speeds. Swinging-bucket rotor is primarily used for rate-zonal centrifugation.

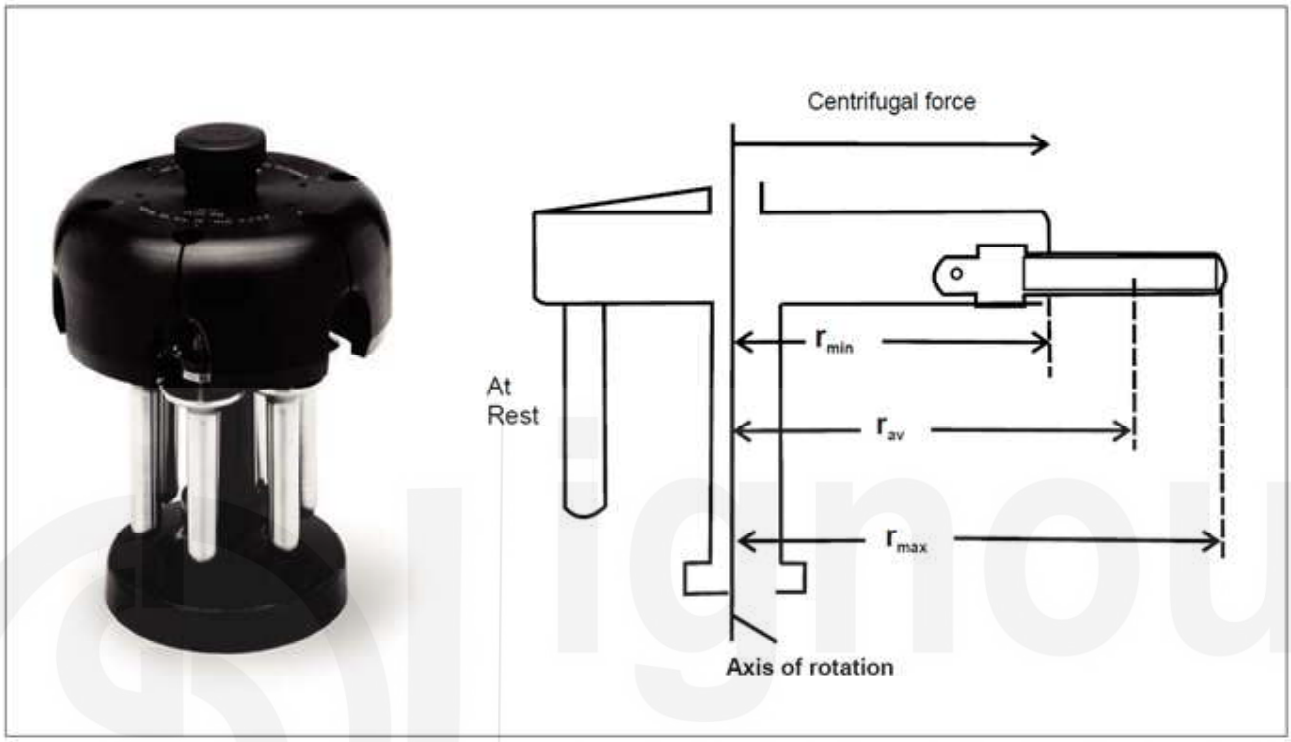


Fig. 4.4: Swinging bucket rotor

Vertical rotor: A vertical rotor is fixed in a vertical position (Fig. 4.5). They are zero angle rotors in which the tubes are aligned vertically in the body of the rotor at all times. They were first introduced in 1970's for high speed and ultra centrifuges. They are best for isopycnic centrifugation and rate zonal centrifugation. They are not used for pelleting as the pellet tends to fall off as the liquid is decanted.

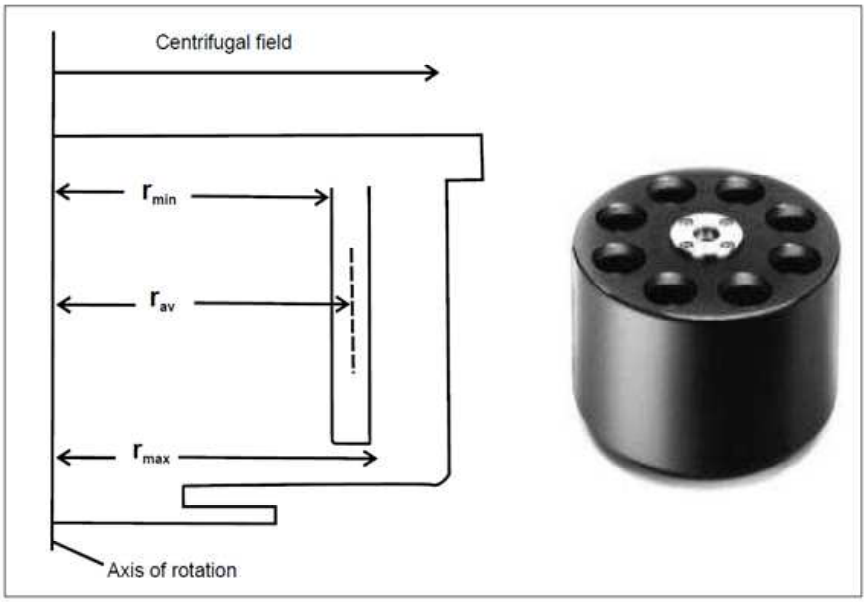


Fig. 4.5 : A Vertical rotor

SAQ 2

Fill in the blanks.

i) High speed rotors are made up of ……

ii) Sample tubes fixed at angle 14º-40º degree in …….

iii) The position of centrifuge tubes is changed from vertical to horizontal position during centrifugation in a ……rotor.

iv) Vertical rotor and fixed- angle rotor are used for …….

v) ……rotor is used for rate-zonal centrifugation.

In the next section you will be introduced to some of the type of centrifuges so that you get an idea of the variations available and their suitability for different purposes.

4.3.2 Type of Centrifuges

All centrifuges are based on the same principle but they differ in the maximum speed of rotation, rotor designs, temperature control and presence or absence of vacuum. We will describe here briefly four major type of centrifuges.

- **Benchtop centrifuge:** Modern benchtop centrifuge (Fig 4.6a) is a low speed (3000- 7000 x g) centrifuge that can be with or without a cooling system. It is routinely used in clinical laboratories for the separation the blood cells, plasma and serum as well as in cell culture applications.
- **Micro centrifuge (microfuge):** Microcentrifuge is widely used for routine laboratory work (Fig 4.6 b). The sample volumes centrifuged are small (0.5 – 1.5ml) and it can accommodate 24 samples. It generates up to 15,000 x g, with or without refrigeration. It is primarily used to spin small centrifuge tubes (Eppendorf) at high speed. It is useful for many biological applications such as pelleting of nucleic acids and for the concentration of proteins samples.

Fig. 4. 6 : (a) A Benchtop centrifuge and (b) A Microfuge

- **Refrigerated centrifuges:** They are refrigerated and can generate high centrifugal force up to 60,000 x g (Fig 4.7). Depending on the rotor sizes

and type, variable number of tubes and volumes can be centrifuged. It is used for differential separation of organelles, separating fractions during partial purification using ammonium sulphate or in obtaining tissue extracts and other temperature sensitive separations.

During high speed centrifugation biological samples can lose structural integrity and can even be irreversibly damaged due to the heat generated, so refrigeration is needed and samples are generally centrifuged at the 4^{o}C.

Fig. 4.7: Refrigerated centrifuge

1. Ultracentrifuges

An ultracentrifuge is the most sophisticated instrument for centrifugation. They are capable of producing high speeds and relative centrifugal field (RCF) of 200,000 x g to 600,000 x g. These centrifuges are equipped with both a cooling system and sealed evacuated chamber, as a lot of heat is generated during high speed ultracentrifugation.

There are two kinds of ultracentrifuges namely, **1. Preparative ultracentrifuge 2. Analytical ultracentrifuge**

In a preparative run all determinations are conducted at the end of centrifugation as it lacks a monitoring system whereas an analytical centrifuge has an optical system for detection of movement of particles during the experiment that depend on either light absorption or they detect changes in refractive index of the solution.

The basic components of a preparative ultracentrifuge includes a drive and speed control panel, temperature control panel, vacuum system, rotor and cooling system (Fig.4.8). For safety reasons, the rotor chamber is completely enclosed in an evacuated heavy armored chamber.

Preparative ultracentrifuge is primarily used in differential and density gradient centrifugation for separation and purification of subcellular organelles, proteins from liquid biological samples.

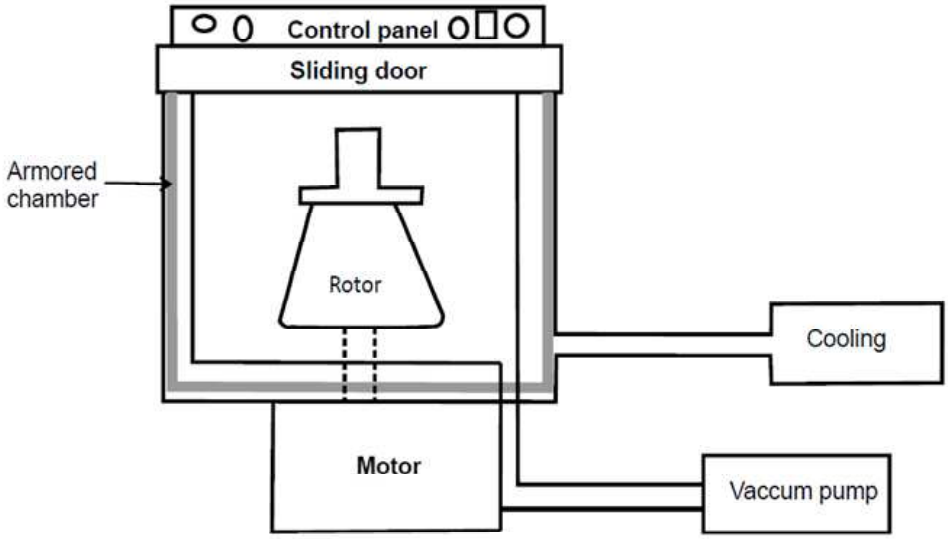
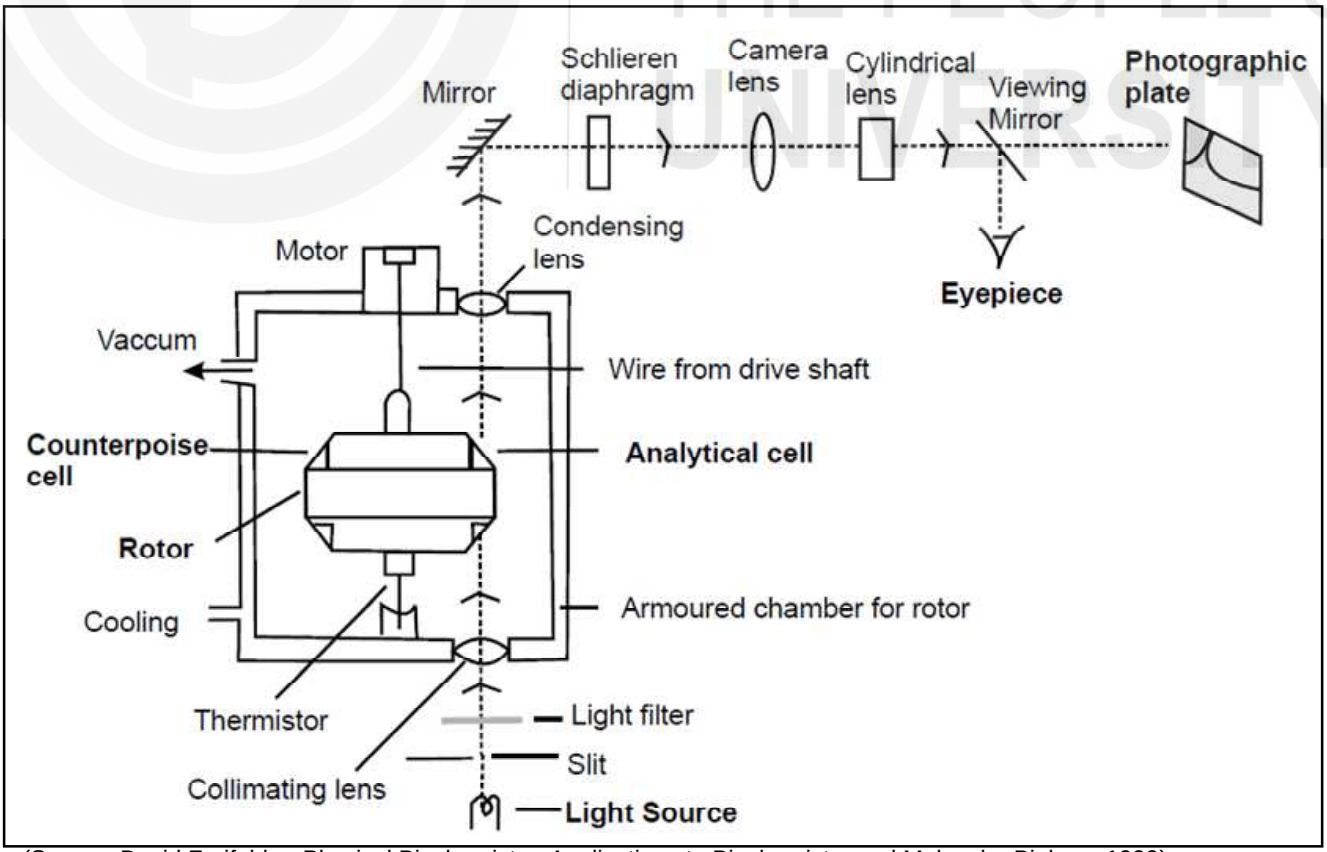


Fig. 4.8: A schematic view of a Preparative ultracentrifuge

An analytical centrifuge is primarily used for determination of sedimentation coefficient and molecular weight of macromolecules such as DNA and conformational changes in protein structure etc. Theodor Svedberg in 1923s developed the analytical ultracentrifuge for studying the protein, haemoglobin and determined the sedimentation coefficient of such molecules. He was awarded with Nobel Prize in 1926 for his work.

The basic parts of analytical ultracentrifuge (Fig.4.9) is similar to the preparative ultracentrifuge except that it is equipped with optical system. It can spin sample at 60,000rpm and an RCF of 600, 000 x g. .



(Source: David Freifelder: Physical Biochemistry: Applications to Biochemistry and Molecular Biology, 1999)

Fig. 4.9: An Analytical ultracentrifuge

SAQ 3

Match the term in column I with that in column II

Column I	Column II
1. Centrifuge equipped with an optical detection system	a. Fixed angle rotor
2. Pelleting heat sensitive samples	b. microcentrifuge
3. pelleting of small samples volumes	c. refrigerated centrifuge
4. Position of centrifuge tubes fixed at an angle of 14°-40°	d. benchtop centrifuge
5. Pelleting of blood cells and serum separation	e. analytical ultracentrifuge

Till now you have learned about the basic concept of centrifugation and instrumentation. Let us how discuss differential and density gradient centrifugation in the next two sections along with their applications.

4.4 DIFFERENTIAL CENTRIFUGATION

Differential centrifugation is a **sequential centrifugation technique** often used for the separation of sub cellular fractionations of organelles from a cell extract. Differential centrifugation is based upon differences in the sedimentation rate of particles of different size, shape and density. In this technique, centrifugation forces are applied in an increasing order for an optimized time period.

When you plan to perform differential centrifugation, a sample solution containing particles of different size and density is prepared in a buffered medium along with stabilizing agents and centrifuged initially at low speed centrifugal force and time period standardized for best fractionation. After centrifugation, the material is separated into two fractions: the **pellet** and the **supernatant** (Fig. 4.10). The pellet is enriched in larger and dense particles and therefore settles to the bottom of the tube. The supernatant is transferred to a fresh centrifuge tube and subjected to next round of centrifugation at a specified centrifugal force that is higher than the preceding speed and given time. This step of centrifugation of the supernatant fraction at an increasingly higher speed for specific time periods followed by separation of the pellet is continued till we get the desired number of fractions / the desired fraction.

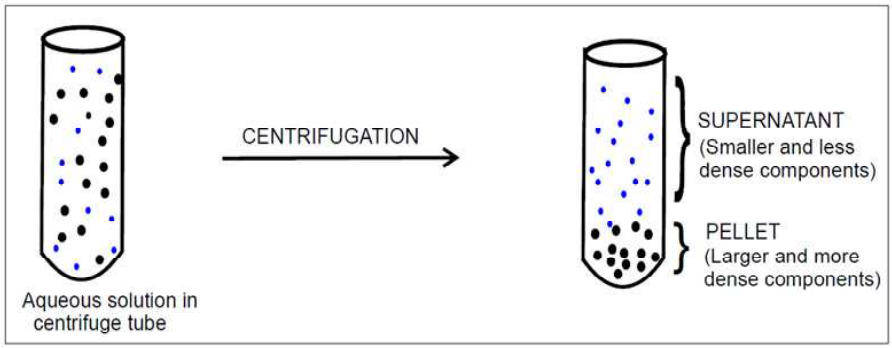


Fig. 4.10: The separation of a sample into a pellet and supernatant fractions

A differential procedure is based on the logic that the largest and densest particles with a higher sedimentation rate require the lowest centrifugal force as compared to the low dense particles. The separation of some of the later fractions (least dense) may require high speed ultra-centrifugation.

This procedure suffers from the inherent limitation of **cross contamination** among the various fractions as absolute fractionation does not generally occur by this method alone. However, washing the pellet with buffers and repeating centrifugation of a given fraction improves the purity of the fraction. Sometimes the fractions are further purified on density gradients or other techniques. The **recovery** is also generally poor.

In section 4.4.1 we will take a specific example of fractionation of cell organelles by differential centrifugation.

4.4.1 Subcellular fractionation

Subcellular fractionation is a technique to separate sub cellular organelles into fractions starting from a tissue homogenate. The first step of fractionation is to separate cells from a tissue and to break open the cells by homogenization. A homogenizer is generally used to facilitate this process. The desired tissue such as rat liver is placed in an **isotonic solution** containing 0.20M sucrose to maintain osmotic pressure. Isotonic solution prevents swelling and bursting of the cellular organelles. The mixture of broken tissue / cells in solution is called tissue **homogenate / cellular suspension** that contains cellular debris, nuclei, mitochondria, lysosomes, ribosomes etc. These organelles differ in size and /or density and therefore, they can be made to sediment at different rates in response to the differential centrifugal force.

Fig. 4.11 depicts schematically a differential centrifugation scheme for separation of cell organelles. A centrifuge tube containing tissue homogenate is placed in rotor and centrifuged at low speed (1000×g for 10 min). The pellet and supernatant fractions obtained following the first round of centrifugation have the nuclear pellet at the bottom of the tube as it is most dense component while the supernatant contains low dense components. The latter is transferred to another tube for further centrifugation at 15,000×g for 15 minutes. Now the pellet is enriched in mitochondria and lysosomes fractions, being the next dense material and remaining supernatant fraction is again centrifuged at 100,000×g for 60 minutes. The pellet fraction of microsomes (membrane vesicles) sediments and smaller dense components remain in supernatant fraction.

The **quality** of the separated fractions is usually assessed by assaying the activity of marker enzymes and sometimes marker lipids / other biomolecules. A **marker enzyme** is an enzyme that is present only in a given organelle. An investigator would ideally expect to get all the marker enzyme activity originally present in the tissue homogenate to be localized in the sub cellular fraction

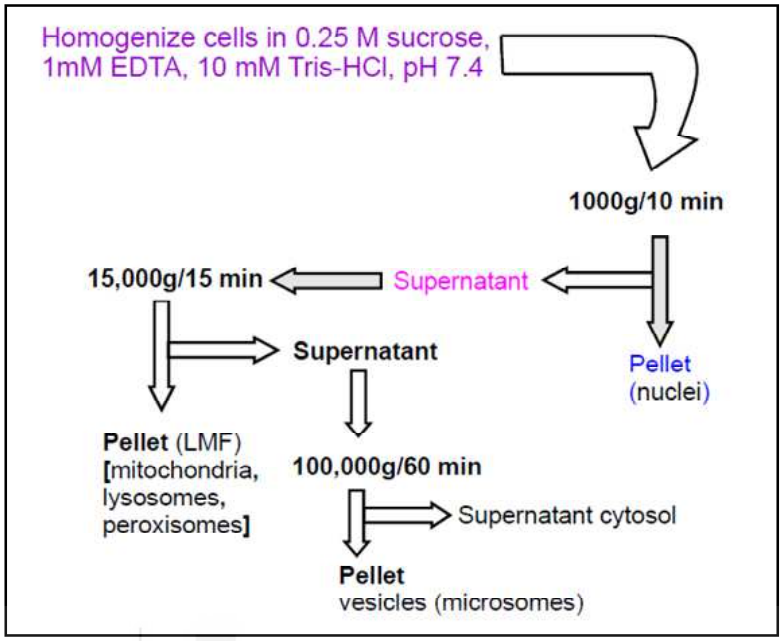


Fig. 4.11: A scheme for subcellular fractionation by differential centrifugation

that has the organelle and absent from all other fractions. The major reason for getting less than expected activity in the desired fraction is due to contamination between fractions. Therefore, to varying degrees the marker activity is also detectable in other fractions. The table below gives the marker enzymes for the major sub cellular fractions.

Table 4.1: Marker enzymes/ other marker molecules in sub cellular compartments

Sub cellular compartment	Marker enzyme / enzyme / another marker biomolecule
Nucleus	DNA / DNA polymerase
Cytosol	Lactate dehydrogenase (LDH)
Mitochondria	Succinate dehydrogenase (SDH)
Lysosomes	Acid phosphatase
Microsomes	Cytochrome P-450

SAQ 4

a) **Define the following terms.**

i) Subcellular fractionation .....

ii) Isotonic solution .....

iii) Tissue homogenization .....

iv) Marker enzymes .....

b) Explain why differential centrifugation is utilised for the fractionation of cell organelles and why do nuclei sediment first among other organelles?

4.5 DENSITY GRADIENT CENTRIFUGATION

As the name suggests, density gradient centrifugation is dependent on the creation of a density gradient of lower (top) to higher (bottom) concentration in the centrifuge tube. Density gradient centrifugation is used to separate particles that differ slightly in size, shape or density. There are two commonly used variants of density gradient centrifugation, namely, **rate-zonal and isopycnic centrifugation**. The density gradient may be established either before particle centrifugation (zonal centrifugation) or during centrifugation (isopycnic centrifugation).

It is generally used to separate and purify cells, sub cellular organelles, nucleic acids, viruses and other biomolecules. This technique provides much better separation than differentiation centrifugation. It is also used along with differential centrifugation to improve the resolution of the various fractions obtained by differential centrifugation of tissue homogenates.

4.5.1 Rate Zonal Centrifugation

Rate-zonal centrifugation resolves different components of the sample into a series of bands or zones on the basis of their sedimentation coefficient. A gradient maker is generally used to prepare a linear **preformed density** gradient.

One of the commonly used chemical to make density gradients is sucrose solution. It is readily available in pure form and does not interfere with most chemical and enzymatic assays. Most such separations use 5% to 20% sucrose or other suitable concentration of sucrose (depending on the length of the tube used) to generate an isokinetic gradient of sucrose. The basic purpose of introducing a steep gradient is to prevent convection due to temperature fluctuations and to prevent mixing due to mechanical disturbances. The technique requires careful layering of the sample on top of a preformed gradient. The density of sample solution to be layered should be less than that at the top of the gradient otherwise it would sink into the density gradient solution.

A sample solution is layered on top of the preformed density gradient in a centrifuge tube that is then placed on a **swinging bucket rotor.** Under the influence of centrifugation field, sample particles move through the density gradient and sediment with gradually decreasing velocity in **narrow zones** depending upon their sedimentation coefficient that in turn is dependent on shape and size of the components. Typical separation of sample particles into three zones is depicted in Fig 4.12. The separated zones can be collected with a needle or syringe from the centrifuge tube.

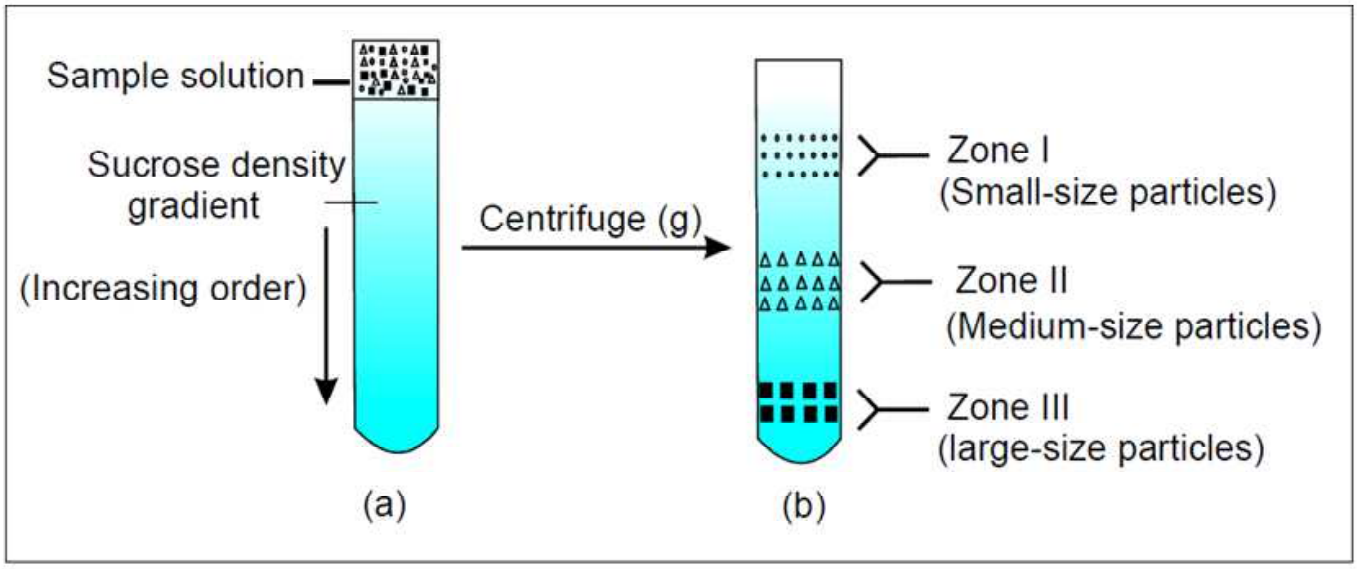


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Fig. 4.12: (a) A sample solution is layered on the top of the preformed density gradient (b) Particles move through gradient solution and separate into zones according to their size, following centrifugation.

It is important to take care that centrifugation must be terminated before any zone reach to the bottom of the tube otherwise the separated bands can be mixed into another zone. Rate zonal centrifugation is performed at **low speed** for a **short time** (incomplete sedimentation). In addition, the path length of the gradient must be sufficient for separation to occur.

It is good for separation of particles of similar density but different masses. This technique has been extensively used for the separation of cellular organelles, RNA - DNA hybrids, ribosomes, ribosomal subunits, polysomes, virus particles and proteins. It is often used for the determination of sedimentation coefficient.

4.5.2 Isopycnic Centrifugation

In isopycnic centrifugation particles separate on the basis of their density rather than size. Particles that have subtle difference in density can be separated by isopycnic centrifugation. The term “isopycnic” means ‘**equal density’** as particles form a **band** at a point where their density and the gradient’s density are matched. This technique is also known as equilibrium density gradient centrifugation**.**

In this technique, a solution of density gradient material such as an alkali metal salt (CsCl) is mixed with the sample and then centrifuged after placing the mixture in a vertical or other suitable rotor. The density gradient is **self-generating** with low density at the top of the tube and increasing densities towards the bottom. The maximum gradient concentration is greater than of the densest species. In order to obtain separation centrifugation is performed at **high speed** and for **long duration.**

Under influence of centrifugal field, particles in the sample migrate through density gradient solution until they reach the position where their **buoyant density** is equal to that of the gradient (isopycnic position). This technique is not dependent on their sedimentation rate or molecular mass. The sedimented

particles in density gradient will form a band. At this point, the particles are at equilibrium and their sedimentation rate is zero. In this technique, sedimented particles will not move into another band if centrifugation time is over and they will never sediment at the bottom of the tube.

The separation of single and double stranded DNA by isopyenic centrifugation is shown in Fig. 4.13. Single stranded DNA has a higher density than double stranded DNA and therefore bands at region of higher density. The classic experiment of Meselson and Stahl used differences in the density of DNA following rounds of replication to demonstrate semi conservative mode of replication. It is also possible to resolve a mixture of DNA, RNA and proteins on the basis of their density. These bands can be isolated from the tube with the hypodermic syringe or needle.

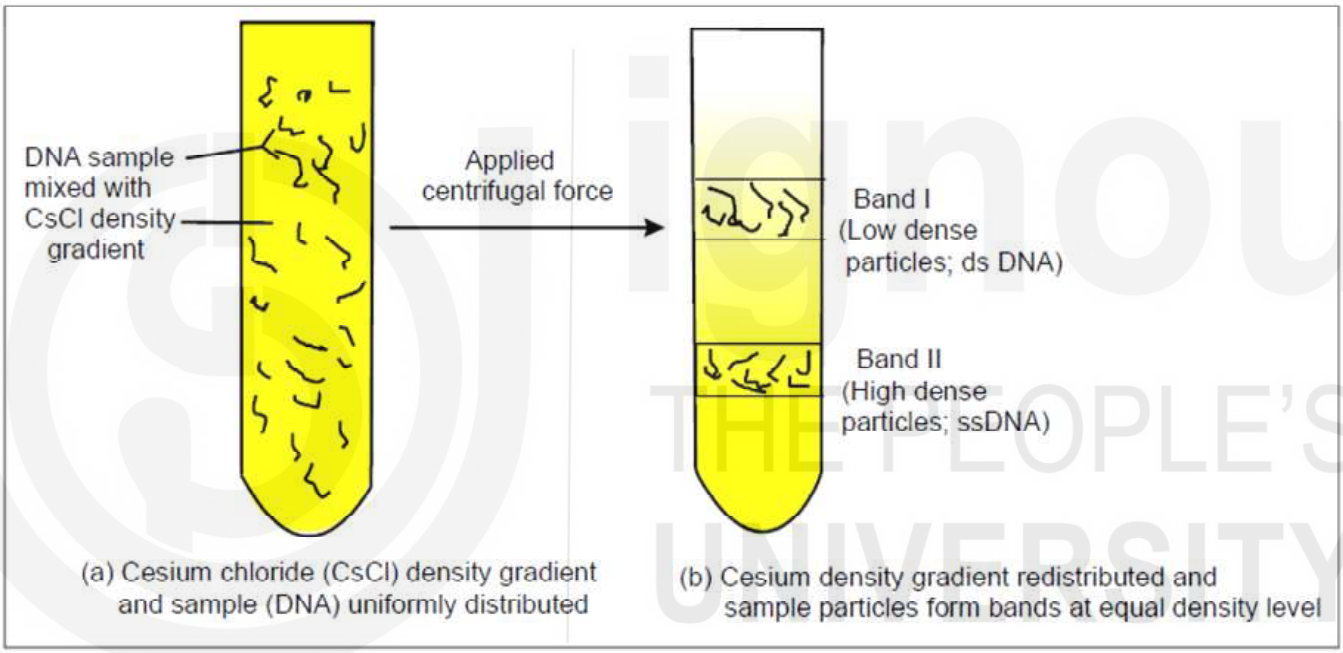

Fig. 4.13: Separation of single & double stranded DNA by Isopycnic centrifugation

This method is generally used for separation and purification of nucleic acids such as DNA and RNA, plasmid DNA and chromosomal DNA, super coiled and relaxed DNA; viruses and lipoproteins. Cesium chloride (CsCl) gradient is generally used to separate DNA molecules whereas Percoll and ficoll gradients are particularly useful for the separation of subcellular organelles.

Density gradient media

The physical characteristics of the density gradient medium determine their suitability biological studies. In general, it is desirable that at the optimal density required the medium should non toxic, recoverable and non-corrosive. It should not penetrate the biological membranes or affect the subsequent biological assays. Table 4.2 summarizes some of the gradient medium commonly used for different purposes.

Table 4.2: Gradient medium for density gradient centrifugation

Density gradient	Uses
Sucrose	Proteins, ribosomes, membrane vesicles
Glycerol	Mammalian cells
Cesium chloride	DNA, viruses, proteins
Ficoll (sucrose polymer with epichlorohydrin) / branched	Mammalian cells such as blood cells, organelles
Percoll (colloidal silica particles coated with polyvinyl pyrrolidone)	Blood cells separation; fractionation of lymphocytes, separation of viable and non-viable cells, virus purification

SAQ 5

a) **In each of the following statements choose the best alternative:**

i) Density gradient centrifugation uses aqueous solution / density gradient medium.

ii) Particles of the similar density but different masses size will separate in rate zonal / isopycnic centrifugation.

iii) Different particles sediment in the different zones / bands in rate zonal centrifugation.

iv) Density gradient medium is prepared in increasing / decreasing order in rate zonal centrifugation.

v) Rate zonal centrifugation / isopycnic centrifugation method is generally performed at high speed and for a long duration.

b) **Define the following terms**

i) Isopycnic density

ii) Density gradient medium

iii) Cesium chloride density gradient centrifugation

c) **Match the term in column I with that in column II**

Column I	Column II
1) Sub cellular fractionation	a) Rate Zonal centrifugation
2) Zonal sedimentation	b) DNA separation
3) Band sedimentation	c) Differential centrifugation
4) Cesium chloride density media	d) Isopycnic centrifugation

4.6 SAFETY MEASURES AND CENTRFIGUE MAINTENANCE

The care of a centrifuge is of paramount importance for its proper functioning, and longevity. In addition, some general safety measures must be followed for all kind of centrifuges

- **Read the manufacture's manual carefully before using the centrifuge.**
- **Select proper operating conditions.**
- **Ensure that opposite tubes are evenly balanced by weighing them, especially when working with high speed centrifuge.**
- **Do not open centrifuge lid during rotation.**
- **Do not touch the rotor in motion.**
- **If you observe shaking, bubbling or excessive vibration sound during centrifugation, stop the centrifuge and contact to manufacturer or dealer.**
- **Rotors and centrifuge should be kept clean and dry during storage.**
- **The laboratory must maintain a log book and every worker must develop the habit of filling the run time and speed used for centrifugation. This is important as all rotors can be best used for a definite number of hours.**
- **Wear a lab coat and face shield or safety goggles while working in the laboratory.**
- **Specific instructions as given by the manufacturer have to be followed for more sophisticated centrifuges.**

4.7 SUMMARY

In this unit, you have learned that:

- Centrifugation techniques play a central role in the separation and purification of cellular components. Particles separate due to differences in size, shape and / density under the influence of applied centrifugal force.
- The Sedimentation coefficient is a constant to define the sedimentation rate of a particle. It is the ratio of sedimentation velocity (v) and centrifugal force (G). It is generally expressed in Svedberg units, where 1 S = 1 x 10^{-13} seconds.
- There are four type of centrifuges that use in different biological applications such as sparation of cells, concentration of samples, purification of subcellular organelles, determination of sedimentation

coefficient. Yet all centrifuges work on the same principle but differ in the maximum speed at which they can be run, rotor designs, temperature control and presence or absence of vacuum.

- Rotor is the main component in the centrifuge. It is the rotating unit that produces centrifugal force on particles to be separated. Rotors are categorized into three types based on the position of tubes relative to the axis of rotation. The three type of rotors are fixed angle, swing bucket rotor and vertical.
- Differential centrifugation is a sequential centrifugation technique that is generally used to fractionate sub cellular organelles.
- In density gradient centrifugation a density gradient is created either before particle centrifugation or during centrifugation. The two varients of density gradient centrifugation are, rate zonal and isopycnic centrifugation. In rate zonal centrifugation particles separate on the basis of size while in isopycnic centrifugation they are separated on the basis of density.
- Every user should familarise themselves with the basic safety measures and maintenance of a centrifuge.

4.8 TERMINAL QUESTIONS

1. State the relationship between centrifugal force and gravitational force.
2. Differentiate between the preparative and the analytical ultracentrifugation.
3. Find out the advantages and limitations of the density media listed in table 4.2.
4. Indicate the differences between:
   a) Density gradient and differential centrifugation.
   b) Rate Zonal and isopycnic centrifugation.
5. Name the types of rotors commonly used in cell biology labs. How will you decide on the type of rotor for your work?
6. What is role of the refrigeration in a centrifuge?
7. Why it is essential to maintain a log book for centrifuges and rotors?
8. Suggest a procedure based on density gradient that can be used for the separation of molecules which differ in density but similar in shape and size.

4.9 ANSWERS

Self-Assessment Questions

1. a) The basic principle of centrifugation depends on sedimentation. Particles separate according to their mass, size, shape and density in an applied centrifugation force. According to the Eqn. (v) :

- Larger dense particles tend to sediment first followed by smaller dense particles.
- The denser the solution, more slowly the particle will move and
- The greater frictional coefficient, the slower the particle will move.

b) Svedberg is a unit to define the sedimentation velocity of a particle in an applied centrifugal force. The 1 Svedberg Unit is expressed by symbol "S" which is equal to 1×10^{-13} seconds.

2. **i) alloys of aluminium or titanium**

ii) Fixed angle rotor

iii) swinging bucket rotor

iv) isopycnic centrifugation

v) swinging bucket

3. 1- e ; 2.- c; 3- b; 4.- a; 5 – d

4. a) i) **Sub cellular fractionation**: A technique used to separate the subcellular organelles from a tissue homogenate. It is generally done by differential centrifugation.

ii) **Isotonic solution**: An isotonic solution refers to two solutions having the same osmotic pressure across the semipermeable membrane.

iii) **Tissue homogenization**: It is the process of disrupting biological membranes and bringing the tissue / cell to a uniform state.

iv) **Marker enzymes**: A marker enzyme is one that is present only in a given organelle. They help to identify a particular organelle by biochemical analysis (Table 4.1).

b) In subcellular fractionation, tissue homogenate is subjected to repeated centrifugation from low to high RCF for different time periods (differential centrifugation). As the cellular organelles differ in size, shape and density so they separate at different RCF.The heavy and large dense components move faster and separate first in pellet fraction followed by smaller and less dense components in sequential centrifugations.

The nuclei sediment first at low centrifugal force as it is the most dense as compared to other cell organelles. Refer to Fig. 4.11 and section 4.4 and 4.4.1 **for details.**

5. a) (I) density gradient medium (i) rate zonal centrifugation (iii) different zones (iv) decreasing order (v) isopycnic centrifugation

b) (i) Isopycnic means "equal'' density. With reference to centrifugation, the samples particles band at a position where their density matches with that of the density gradient medium.

(ii) Density gradient media are chemicals that are used to create a density gradient along the length of the centrifuge tube. The gradient may be either preformed or self generating. Refer to table 4.2 for commonly used density gradient media.

(iii) CsCl density gradient centrifugation is an example of isopycnic centrifugation. It is generally used for separating and purifying nucleic acids that differ in density.

c) **1. - c; 2. - a; 3. - d; 4. - b; .**

Terminal Questions

1. The relationship between centrifugal force and gravitational force acting on a particle is expressed by Relative Centrifugal Field (RCF).

$$\mathbf{RCF = (1.12 \times 10^{-5}).\ (rpm)^2 \times r}$$

It is the ratio of the weight of a particle in an applied centrifugal field (G) to the weight of a same particle when acted by gravity alone.

It is important to remember that RCF is measured in units of gravity or × g) and speed of rotation of a particle is expressed as revolutions per minutes (rpm).

2.

Analytical ultracentrifugation	**Preparative ultracentrifugation**
Equipped with optical system for detection of movement of particles.	Lacks a monitoring system / All determinations are conducted at the end of the experiment.
Used to determine the sedimentation coefficient and molecular weight of the purified macromolecules such as protein, DNA, plasmid and subcellular organelles.	Mostly used for separation and purification of sub-cellular organelles, nucleic acids, proteins and other particles to biochemical interest.

3. Advantage: Separation and purification of desired molecule and cell organelles are possible using the density gradient.

Disadvantage / limitation: Poor quality of density gradient media can react to cellular components. Some can penetrate the biological membranes or affect subsequent biological assays. The students should find out the specific advantages and disadvantages of few density gradient media referred in table 4.2.

4. a)

Density gradient centrifugation	**Differential centrifugation**
It involves a single round of centrifugation that is performed either at low or high speed.	It is a sequential centrifugation technique and requires multiple centrifugation steps of incrementally increasing centrifugal force.

Sample applied on top of a preformed gradient or the gradient is self generating.	A density gradient is not needed or the run.
Particles separate in zones (rate zonal) or bands (isopycnic centrifugation) according to their mass or buoyant density, respectively.	Particles separate into pellet and supernatant fractions according to their differences in sedimentation rate of particles that is dependent on the is size and density.
Used to separation of cellular organelles, RNA - DNA hybrids, ribosomes, ribosomal subunits, polysomes, virus particles and proteins.	Generally used for fractionation of sub cellular organelles.
It is possible to obtain much purer separations.	Purity of fractions is poor due to the cross contamination.

b)

Rate-Zonal centrifugation	**Isopycnic centrifugation**
Separation of particles is based on the sedimentation coefficient of . particles. It is good for particles of similar density but different masses	Separation is based on particle density and sedimentation equilibrium. It is not dependent on sedimentation rate or molecular mass.
Sample is placed on top of the preformed density gradient	Sample is mixed with the density gradient medium / self generating gradient
Low speed, proper time limit / incomplete sedimentation	High speed, longer running time / complete run until equilibrium is reached.
Used to separate cell organelles, RNA-DNA hybrids and ribosomal subunits.	Used for separation and purification of supercoiled DNA, plasmids, viruses, lysosomes, mitochondria and proteins etc

5. There are three types of centrifuge rotors based on the position of tubes relative to the axis of rotation.They are:

   1. **Fixed- angle rotor** : useful for differential centrifugation (subcellular fractionation), and density gradient centrifugation for the separation of cellular organelles, virus particles, etc.
   2. **Swinging-bucket rotor**: primarily used for rate-zonal centrifugation and pelleting of cellular fractions.
   3. **Vertical rotor**: suitable for isopycnic centrifugation and rate zonal centrifugation.

6. Refrigerated system helps to maintain a near constant temetrature by counteracting the excess heat (high temperature) generated during high speed centrifugation. This is important in case of biological materials as high temperatures may lead to loss of activity due to irreversible denaturation or other temperature dependent changes.
7. A log book is meant to keep a record of the use of rotors and the duration and speed of centrifugation. This is important as all rotors can be best used for a definite number of hours. A log book also records the working condition of the instrument since it was last used.
8. Isopycnic centrifugation can be used to separate molecules which differ in their density but have similar molecular mass. Refer to subsection 4.5.2.

4.10 SUGGESTED READINGS

1. Wilson and Walker Principle and Techniques of Biochemistry and Molecular Biology. 7th Edition, 2010. Canbridge University Press.
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3. Upathya and Upathaya, Biophysical Chemistry, Principle and Techniques, 6th Edition, 2002. Himalayan Publishing House.
4. David Freifelder. Physical Biochemistry; Application to Biochemistry and Molecular Biology, 2nd Edition 1999, W.H. Freedom and Company.