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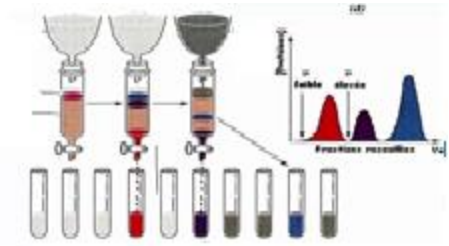
University of Science and Technology of Oran
- Mohamed BOUDIAF -
Faculty of Natural and Life Sciences
Department of Basic Studies in Biology

Polycopy of the course

SCIENTIFICS METHODOLOGY AND TECHNIQUES FOR THE STUDY OF LIVING

For Second-Year Common Core Students
Field: Biological Sciences and Biotechnology

Rrepared by Dr. Soraya AINAD-TABET



Course Identification

Course Title : Scientific Methodology and Techniques for Studying Living

Semester : **S4**

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Number of Credits : **04**

Coefficient : **02**

Total hours per semester : 45h (15 weeks)

Volume horaire hebdomadaire :

- Lectures (number of hours per week) : 01h30
- Practical work (number of hours per week) : 1h30
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University of Science and Technology of Oran "Mohamed BOUDIAF"

Faculty of Natural and Life Sciences

Department of Biotechnology

Assessment Methods

Nature of the control	Partial weighting in %	Weighting in %
Exam	/	60
Test	/	40
Tutorials	/	
Practical work	/	
Personal project	/	
Group work	/	
Field trips	/	
Assiduity (Presence or Absence)	/	
Other (to be specifed)	/////////	
Total	100%	**100%**

Disclaimer

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- ✓ Improve the structure and flow of the text
- ✓ Refine scientific explanations and terminology
- ✓ Format references according to academic standards
- ✓ Ensure consistency across chapters
- ✓ Facilitate the translation of this document

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Introduction

The scientific method refers to a set of structured approaches aimed at guiding the process of producing scientific knowledge. It encompasses observation, experimentation, reasoning, and problem-solving to understand phenomena and achieve specific objectives within the framework of scientific research through investigations.

The study of the practices of researchers, however, reveals such a great diversity of scientific approaches and disciplines that the idea of a unified method becomes highly problematic.

As for technique, it refers to all the tools, instruments and means specific to an activity or research. All scientific research is based on the formulation of hypotheses, their verification through observation and experimentation, as well as their validation.

The verification phase relies on specific tools, instruments and means for each research project, which are called «Techniques».

Course description

Objective :

This subject enables students to acquire knowledge of the methods used for studying living organisms, particularly cytological methods, techniques for analyzing the biochemical composition of cells, and approaches specific to living organisms.

Syllabus

First Part: Methods for Studying Cell

Chapter I: Cytological Methods

Chapter II: Methods for Studying the Biochemical Composition of Cells

Chapter III: Cytochemical and Immunological Methods

Chapter IV: The methods of genetic engineering

Second Part: Methods and Techniques for Studying Living Organisms

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Part One: Methods for studying cell morphology

Chapter I: Cytological Methods

Cells, being small and complex, require advanced tools to study their structure, chemical composition and function. Thanks to advances in microscopy, particularly electron microscopy, it is now possible to observe even individual atoms in crystals.

Indispensable in modern medicine and biology, the microscope allows us to explore the field of cellular biology. To achieve this, it is crucial to understand the techniques and devices used. Three specific approaches have been developed to analyse the various aspects of cells.

- Morphological techniques
- Chemical and biochemical techniques
- Physiological techniques

These approaches rely on the use of optical and electron microscopes.

This chapter presents the main techniques of light microscopy as well as the methods of electron microscopy used for observing biological samples. It also covers the various preparation stages that samples must undergo before observation, which are adapted according to the nature of the specimen studied and the type of information sought.

1. Microscopy

Microscopy encompasses techniques that allow visualization of structures at the microscopic scale. Its principle is based on directing a wave onto a specimen, followed by its reception by an objective lens that focuses it. The wave then passes through an eyepiece which generates a visible image. This image can be observed directly, photographed, or recorded via a camera for subsequent computer processing.

An optical microscope is an optical instrument equipped with a lens and an eyepiece that magnifies the image of a small object (Figure 1).

The primary objective of a microscope is to reveal the microscopic structure of objects, allowing the distinction of fine details rather than simply enlarging them. The essential

performance of this instrument lies in its resolution, that is, its ability to separate and distinguish these details.

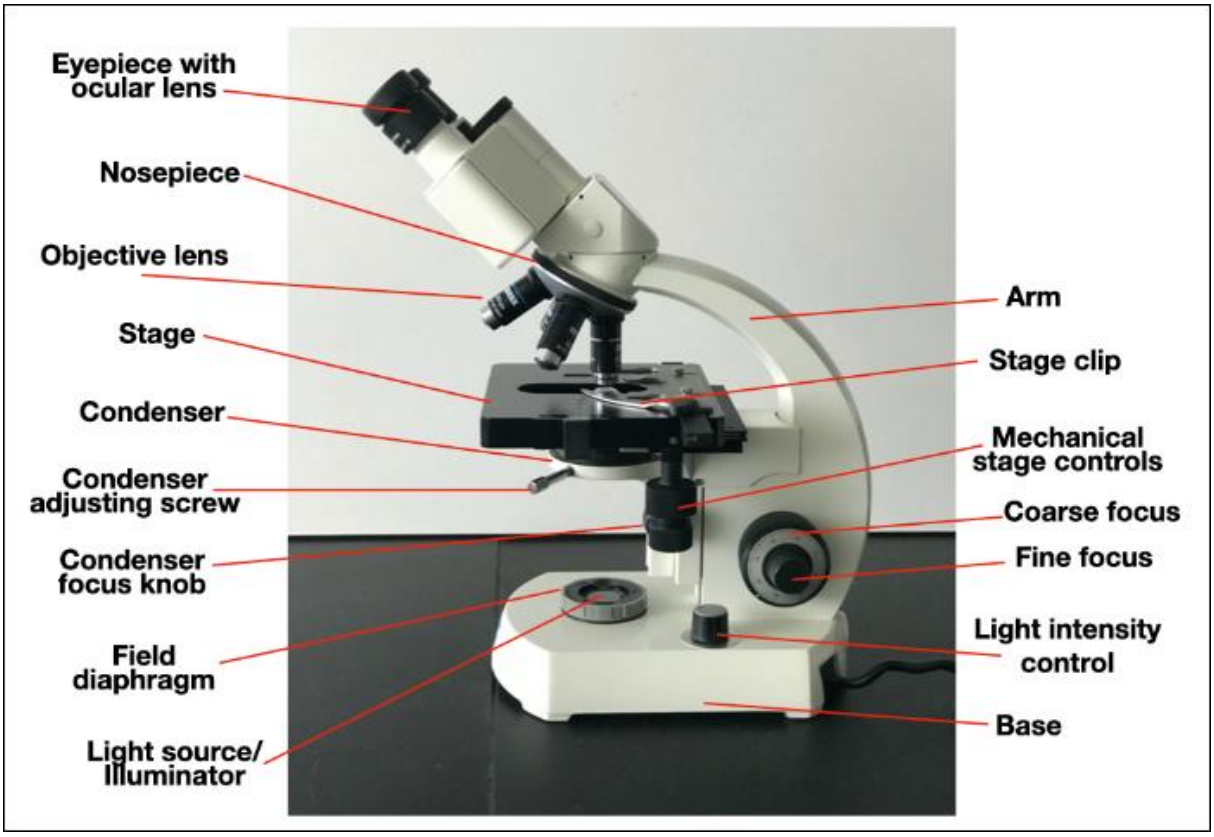


Figure 1: Optical microscope

How does the optical microscope work?

The three main functions of a microscope are:

- To provide a magnified image of a small object (magnification).
- To separate its details in the image (resolution).
- To make the details visible to the eye or with the help of a camera.

Principle:

A wave is sent onto a specimen. This wave is captured by an objective lens (which produces a real, enlarged, and inverted image placed in front of F2). The image then passes through the eyepiece (which produces an enlarged and inverted virtual image) that creates the final observable image (Figure 2).

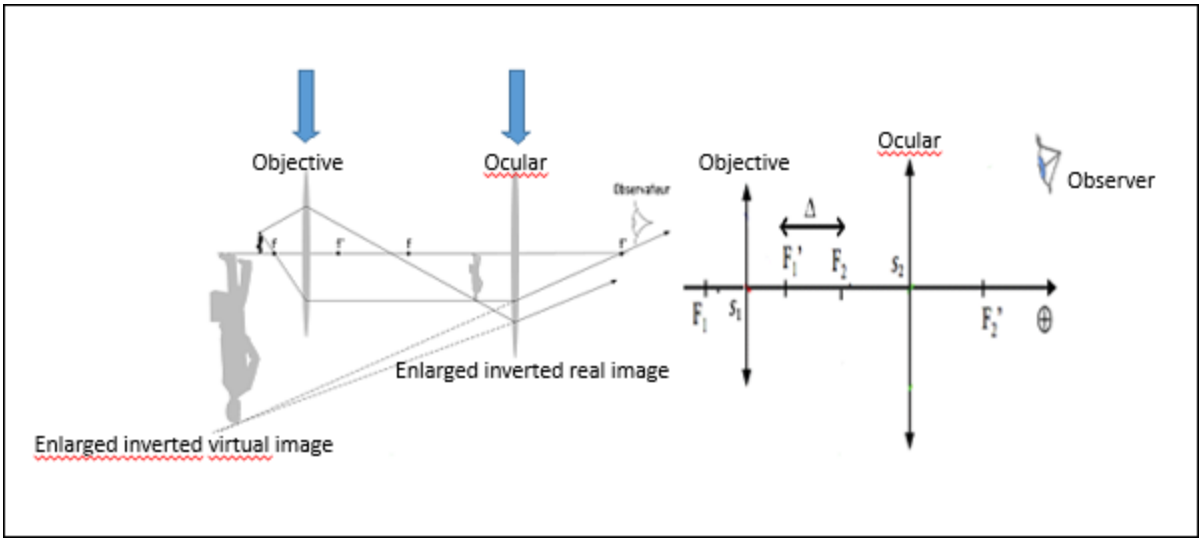


Figure 2: Physical working principle of the microscope

What characterizes a microscope?

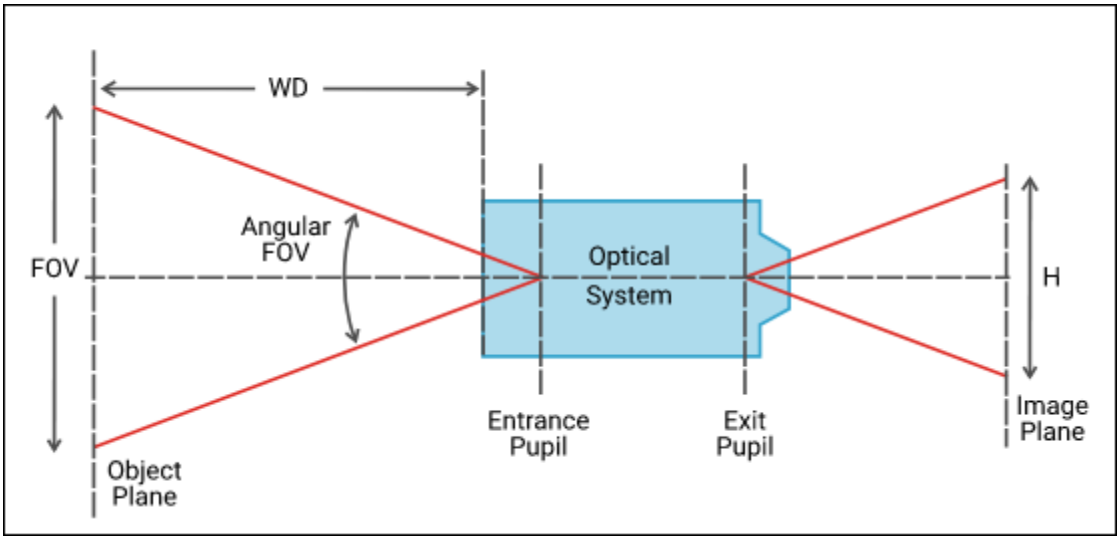
1.a- Magnification: The final magnification corresponds to the product of the magnifications of the two glass lenses. The total magnification (Gr) of the microscope is equal to the product of the objective's magnification (a ratio of lengths) and the eyepiece's magnification (an angular ratio).

Gr = Magnification (objective) x grandissement (ocular)

2.b- Working distance: The working distance refers to the space between the microscope objective and the specimen being observed. A shorter working distance generally indicates higher magnification, allowing for more detailed and sharper images (Figure 3).

3.c- Resolving power (resolution): Resolution is the smallest distance between two objects that can be distinguished using the microscope. This resolving power depends both on the wavelength of the radiation and on the quality of the objective lenses. Under optimal conditions, the resolving power of a light (optical) microscope reaches its theoretical limit, which ranges between 0.2 and 0.3 µm. It can be increased by placing a drop of immersion oil (a colorless oil in which light travels at the same speed as in glass) on the specimen (De Robert, 1983). This is

called "oil immersion" observation, where the oil replaces the air between the object and the objective.



Working Distance (WD), Field of View (FOV), Angular Field of View (AFOV), the sensor size (H).

Figure 3: Explanatory diagram of the working distance in a microscope

Detection techniques now make it possible to accurately identify all kinds of molecules, and photographs (on film or digital) can be analyzed for quantitative studies (size, number, and location of the observed elements). Today, microscopy is divided into two major groups, distinguished by the nature of the elementary particle involved:

- The optical microscope, also called photonic, because it uses photons,
- The electron microscope, which uses electrons to study the specimen.

1.1- The Optical Microscope

In this type of microscope, a lamp illuminates the specimen. The molecules to be observed interact with light in several ways:

- Either by absorbing certain wavelengths of light. This is bright-field microscopy.
- Or by causing a phase shift of the different light rays. This is phase-contrast microscopy.
- Or by emitting light at a different wavelength than the original one. This is fluorescence microscopy.

What are the different types of optical (or photonic) microscopes?

There are several types of optical microscopy:

• Bright-field (transmission) microscopy
• Phase-contrast microscopy
• Dark-field microscopy
• Polarized light microscopy
• UV microscopy (fluorescence microscopy)
• Scanning microscopy

1.1.1- Transmission or Bright-Field Microscopes

The optical microscope using transmitted light makes it possible to study fixed and stained cells. It uses visible light (white light composed of several wavelengths).

The white light emitted by a lamp is concentrated onto the biological specimen and passes through it. Depending on the intensity of the staining, the light will be absorbed to a greater or lesser extent, and the area will appear darker or lighter, while weakly stained regions remain relatively clear.

These microscopes are equipped with a system of lenses that condense the light onto the specimen to be observed.

Observation of biological samples using transmission light microscopy requires preparation steps intended to make the sample:

• thin enough for light to pass through it (sectioning).
• contrasted enough to absorb more light than the surrounding medium (staining).

1.1.2- Phase-Contrast Microscope

This technique allows the observation of cells without preparation or staining, in their original medium. It is therefore one of the rare methods that makes it possible to observe living cells. For this reason, it is widely used in cell engineering.

The principle is based on the fact that biological structures are transparent. This technique converts differences in refractive index within the sample into differences in light intensity. The principle consists of illuminating the sample with a ring of light (obtained by placing a ring-shaped diaphragm in the condenser) and collecting the light with a special objective lens containing a phase plate-that is, a plate with a ring (corresponding to the image of the condenser ring formed by the objective) that produces a phase shift in the transmitted light (Figure 4). This type of microscope is widely used to observe cell movements and to follow the stages of cellular processes, for example mitosis. It is also extensively used in bacteriology because of its ability to reveal poorly opaque objects (Figure 5).

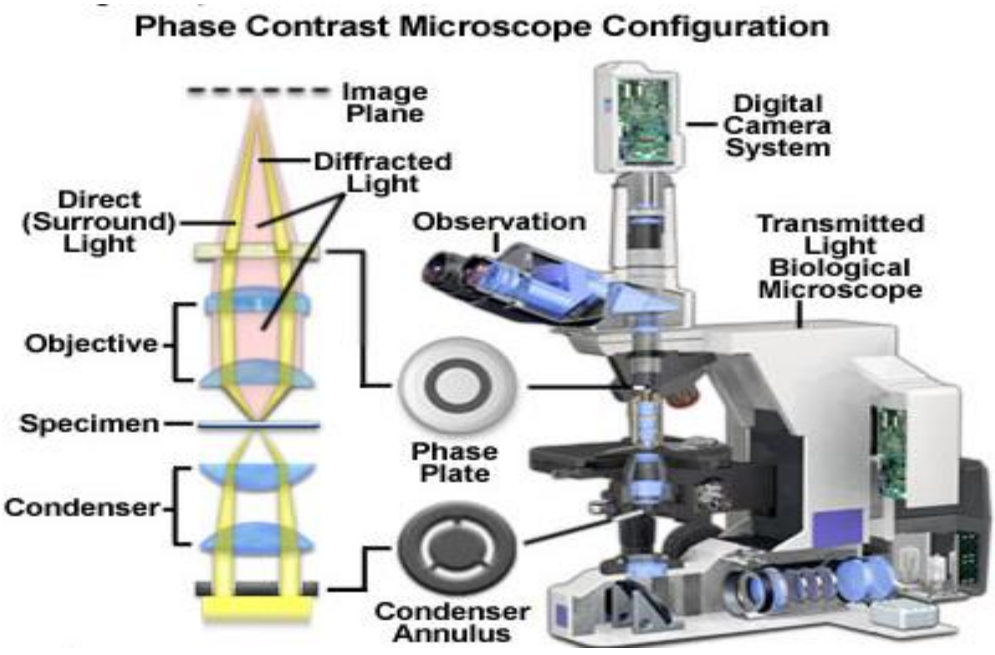


Figure 4: Phase-contrast microscope

Figure 5: Euglena photographed with a 40X Motic Phase objective. The long flagellum is about 0.5 microns (500 nm) thick (Robert Berdan, 2017)

1.1.3- Dark-Field Microscope

The dark-field microscope (also called dark-field microscopy) uses light rays against a dark background. The areas of the specimen that scatter light appear bright against this black background. It is used for elements that are too transparent to be clearly seen with bright-field microscopy. The observed structures appear very luminous (such as flagella and fibers).

It is not used for observing stained or colored objects. Dark-field microscopy is particularly suitable for unstained samples. It allows the observation of living and moving structures such as bacteria or unicellular organisms and even multicellular organisms like pollen (Figure 6).

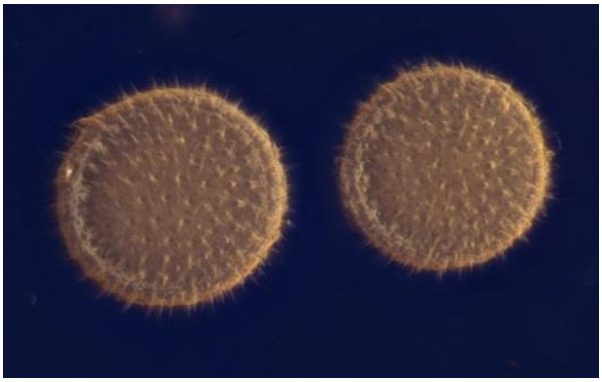


Figure 6: Pollen of *Malva* Grx40 (Daniel Nardin, 2014)

1.1.4- Polarized Light Microscope

The polarized light microscope allows the detection of birefringent structures that have a specific molecular organization, such as microtubules, chloroplasts, and plant cell walls. It is

therefore used in petrography to observe and identify minerals in rocks. A polarized light microscope is an optical microscope whose technology relies on the use of a polarized light beam (light waves vibrating in a single direction). To polarize the light, a polarizer is placed after the light source and before the specimen. The second polarizer, called the analyzer, is positioned after the specimen and perpendicular to the first polarizer, so it blocks the initially polarized light (Figure 7).

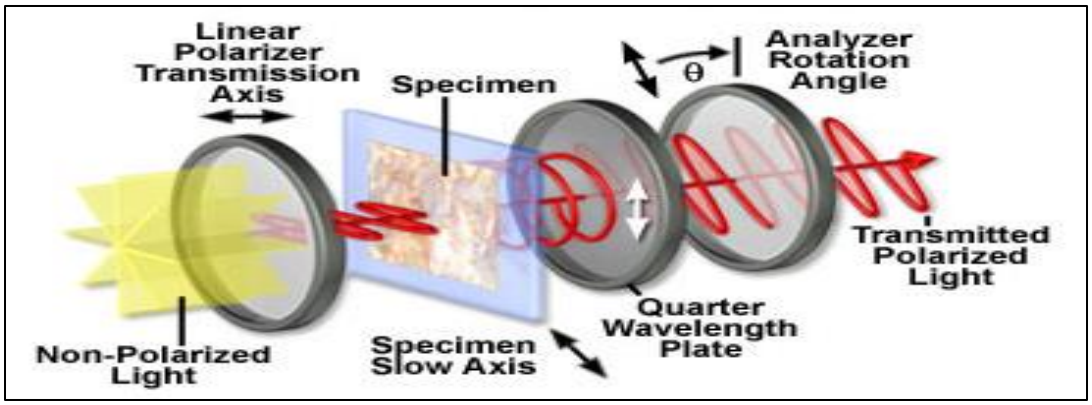


Figure 7: Principle of Operation of the Polarizing Microscope (Michael & Davidson; 2015)

1.1.5- UV Microscope (Fluorescence Microscope)

This technique is used for certain molecules that emit light after absorbing photons of a shorter wavelength. Fluorescence microscopy is based on forming an image by detecting this emitted light (Murphy and Davidson, 2012). The specimen is illuminated with ultraviolet (UV), violet, or blue light. Fluorescence microscopy typically involves the use of a fluorescent dye to highlight specific structures (Figure 8).

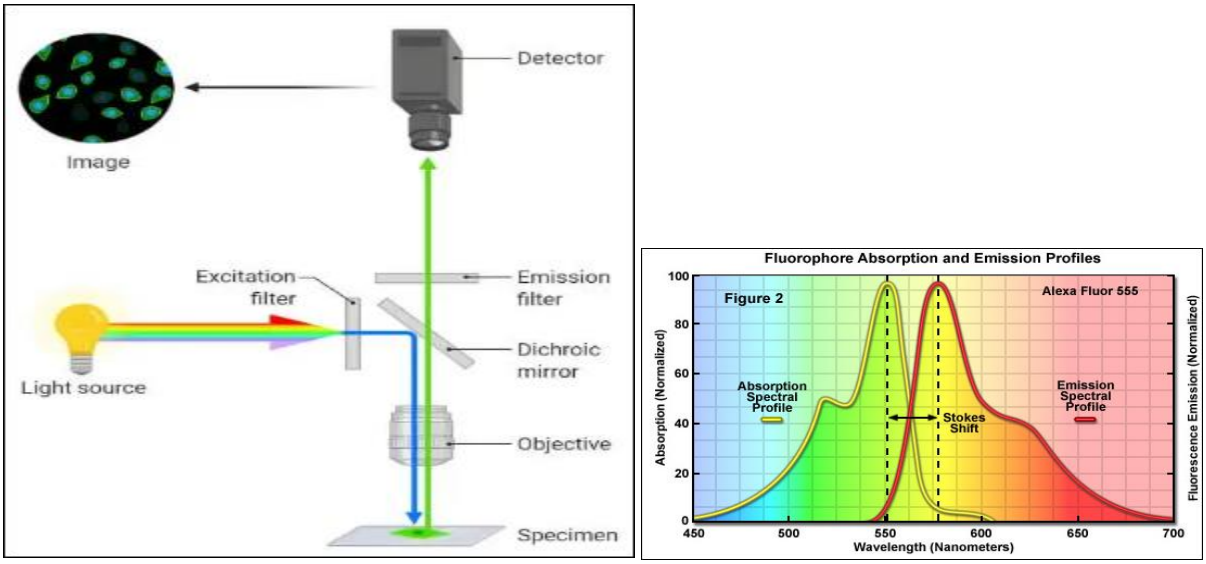


Figure 8: Explanatory diagram of the working distance in the optical microscope (Prakriti, 2024)

It is very useful for analyzing both naturally fluorescent substances (such as chlorophyll) and artificially labeled fluorescent substances attached to molecules, called fluorochromes or fluorophores**,** such as rhodamine (emits red light) and fluorescein (emits green light). In fluorescence microscopy, it is possible to directly visualize naturally fluorescent substances, such as chlorophyll, which absorbs light at 430 nm (blue) and emits green light.

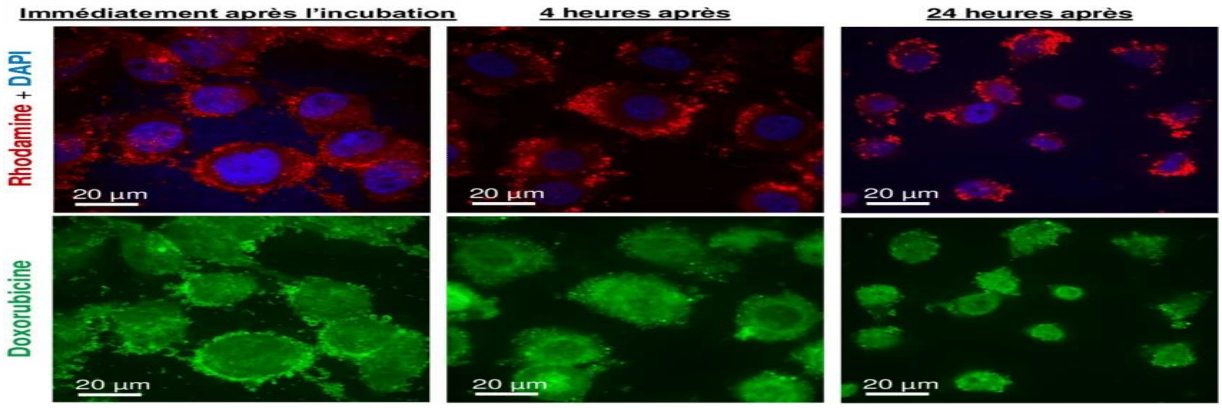


Figure 9: Confocal fluorescence microscopy images showing the internalization of DOXMagNanoGels-37% in PC-3 cancer cells: immediately (left), 4 hours (center), or 24 hours (right) after incubation. The DOX-Mag Nano Gels are covalently bound to rhodamine (RHO, red) and loaded with doxorubicin (DOX, green). The cell nuclei are stained with DAPI (blue) (Cazares cotes, 2017)

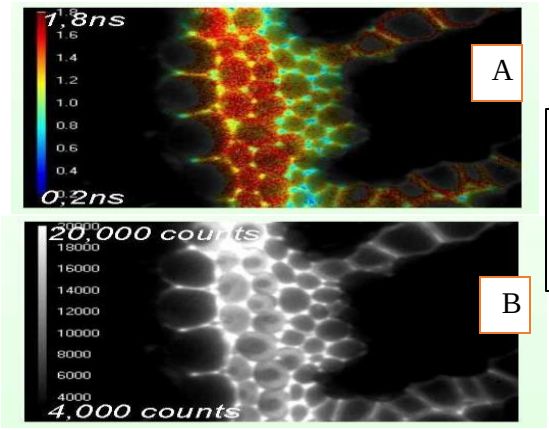


Figure 10 : In these figures, animal cells were stained with a fluorochrome solution. You can distinguish between tissue labeled with fluorophores (**A**) and natural, unstained tissue (**B**).

1.1.6- Scanning Microscope

In this type of microscope, a finely focused laser beam that rapidly scans a single plane of the specimen, illuminating only a thin section, these are called optical sections, illuminates the specimen. Staining is done with fluorescent probes, and the light emitted from the illuminated optical section is used to produce an image on a video screen. This type of microscope is used for observing thick samples. Photographic sections of a human brain taken by a scanner are an example of this technique.

This technique is effective for both fixed and dynamic observations. Furthermore, it is the most suitable microscopy method for thick samples due to its high resolution. It is also used to observe surface topography, providing information on the structure and texture of a specimen and producing images that are close to three-dimensional representations.

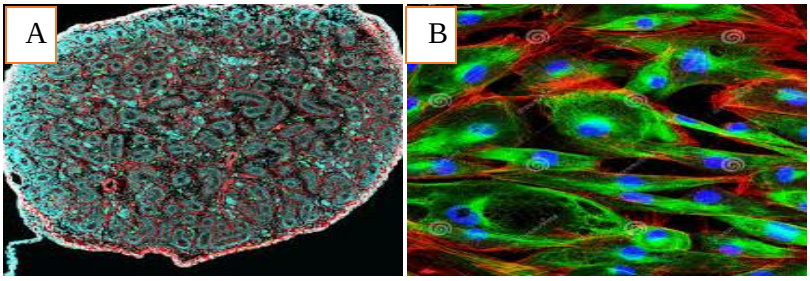


Figure 11: Observation of human organ tissue: testis **(A)** and fibroblast **(B)** under a scanning microscope

1.2- Advantages and limitations of photonic microscopes

This section presents the different types of photonic microscopes, each suited to specific observational purposes. Table 1 summarizes their main advantages and limitations, helping users choose the most appropriate microscope according to their experimental needs.

Table 1: Advantages and limitations of light microscopes

Types of photonic microscopes	Advantages	Limitations
White Light Microscope	Simple and easy to handle; provides a general view of the cell.	Low contrast of observed cells. Tissue contrast can be increased without staining.
Phase Contrast Microscope	Highly effective technique for transparent biological specimens with a refractive index close to that of the surrounding medium.	Not suitable for thick or stained specimens such as tissue sections.
Dark Field Microscope	Live observation of unstained samples without the need for complex sample preparation.	Reduced resolution and potential for artifacts due to scattered light.
Polarized Light Microscope	High performance and precision for imaging certain cellular structures: fibers, collagen, etc.	More specialized application in materials chemistry and rarely used in biology due to the specific nature of its illumination.
Fluorescence Microscope	Widely used in cytochemistry; the best method for detecting the localization of molecules.	Image blurring due to out-of-focus emitted fluorescence.

1.3- Electron Microscope

The electron microscope is much more recent compared to the optical microscope. The possible magnifications range from 1,000× to 50,000×. Its resolution can reach up to 2 angstroms.

How the electron microscope works?

Electron microscopy uses a beam of electrons instead of light. Indeed, the sample to be observed is bombarded with a stream of electrons rather than illuminated by light. This electron beam has a much shorter wavelength than visible light, resulting in a much higher resolution,

reaching the scale of atomic structures. The image is made visible on a fluorescent screen; these images can be photographed and are often further enlarged afterward.

With this microscope, only dead cells can be examined; however, its resolving power is on the order of a few angstroms. Therefore, it allows access to the ultrastructure of organelles.

What are the different types of electron microscopes?

There are two types of electron microscopy:

- Transmission electron microscopy
- Scanning electron microscopy

1.3.1- Transmission Electron Microscope (TEM)

This is the most powerful technique. In principle, it is similar to direct light optical microscopy. The electron beam is emitted by an electron gun, focused onto the specimen using electromagnetic lenses, and passes through it. The electrons are more or less absorbed, and the image is formed behind the specimen on a fluorescent screen. Apart from the fact that electron-absorbing agents are heavy metals, the same staining techniques used in direct light microscopy can be applied (Figure 12).

Figure 12: Transmission electron microscope

This technique requires the use of fixed (non-living) samples. The steps are as follows:

- Chemical fixation

- Dehydration
- Embedding in resin
- Ultramicrotomy: ultra-thin sectioning
- Staining of the sample
- Observation of the section

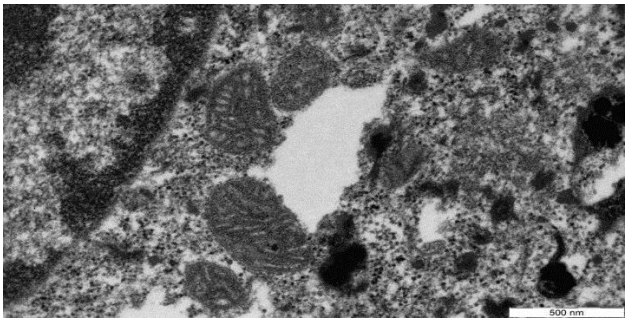


Figure 13: Mitochondria in melanized observed by transmission electron microscopy (500nm)

1.3.2- Scanning Electron Microscope

The scanning electron microscope is a tool for observing surface topography. It provides information about the structure and texture of a sample and produces absolutely spectacular pseudo-3D images. The electron beam scans the surface of the specimen, which is pre-coated with a thin metallic layer. Secondary electrons, reflected from the metallic surface, are used to generate the image. This instrument offers increased depth of field, but its resolving power is lower than that of the transmission electron microscope (Figure 14).

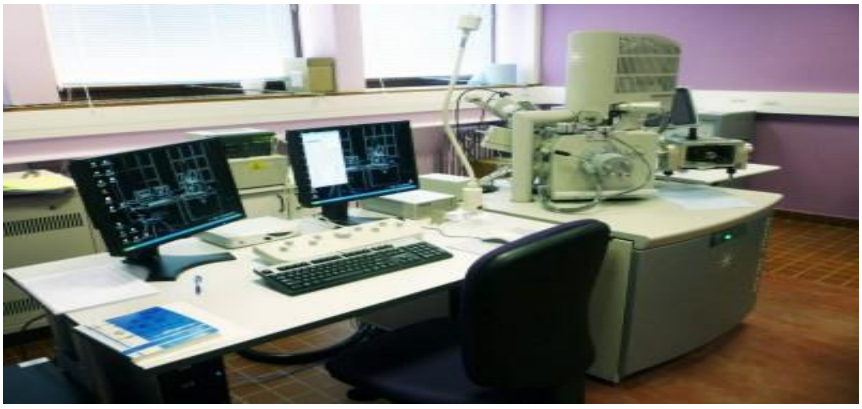


Figure 14: Scanning electron microscope

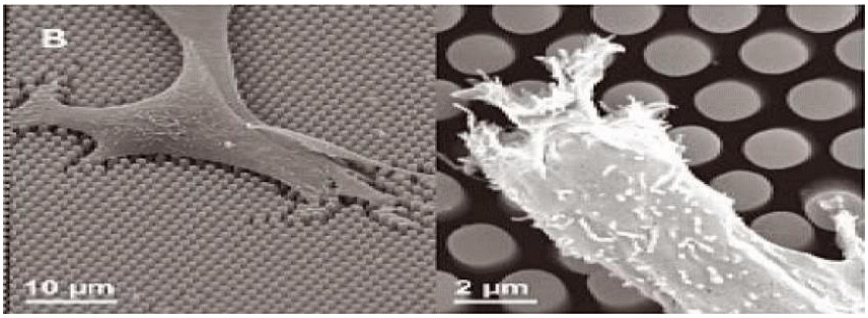


Figure 15 : Scanning electron microscopy images of cells cultured on deformable micropillar arrays. The cells rest on top of the pillars without penetrating between the posts and exert forces detected by the deflection of the pillars (Saez, 2007)

Its principle is based on increasing contrast (not of the specimen itself, but of the space surrounding it) by using electron-impermeable substances such as phosphotungstic acid. Since the samples must be placed under vacuum for observation, biological specimens that are rich in water must first be dehydrated. The samples are frozen, and then the ice is sublimated under vacuum.

This technique requires the use of fixed (non-living) specimens. The steps involved are as follows:

- Freezing
- Cryofracture
- Replica preparation
- Observation of the specimen.

2- Comparison between the optical microscope and the electron microscope

The main difference between the optical microscope and the electron microscope lies in their method of illumination and magnification. The optical microscope uses visible light to illuminate the specimen under study, whereas the electron microscope uses a beam of electrons for illumination. In addition, the optical microscope can magnify up to about 1000×, while the electron microscope can achieve magnifications exceeding 100,000×. Optical microscopes are generally more affordable and more portable than electron microscopes, as they are typically less complex and smaller in size. Each type has its own advantages and disadvantages (Table 2).

Table 2: Comparison between the optical microscope and the electron microscope

S.No	Light microscope	Electron microscope
1.	Light is the illuminating source	The beam of electrons is the electron source
2.	Specimen preparation takes usually few minutes to hours. Live or dead specimen may be seen	Specimen preparation takes usually takes a few days. Only dead or dried specimen are seen
3.	Condenser, objective and eye piece lenses are made up of glasses	All lenses are electromagnetic
4.	Specimen is stained by coloured dyes	Specimen is coated with heavy metals in order to reflect electrons
5.	It has low resolving power (0.25µm to 0.3 µm). It has a magnification of 500X to 1500X	It has high resolving power (0.001µm), about 250 times higher than light microscope. It has a magnification more than 100,000X
6.	Vacuum is not required	Vacuum is essential for its operation
7.	Image is seen by eyes through ocular lens	Image is produced on fluorescent screen or photographic plate

Chapter II. Methods for studying the biochemical composition of cells

These methods make it possible to determine the nature and localization of the biochemical constituents of the cell.

1- Cellular materials

The cell is the basic structural and functional unit of biological organisms.

1.1- Different types of cells

There are two fundamental types of cells:

- **Prokaryotic cells (pro = primitive; karyon = nucleus):** Cells without a true nucleus, meaning that the genetic material is not enclosed within a nuclear envelope, and lacking membrane-bound organelles.

- **Eukaryotic cells (eu = true, karyon = nucleus):** the nucleus is bounded by a nuclear envelope. Internal membranes define cytoplasmic compartments called organelles. Among eukaryotic cells, two types can be distinguished: animal and plant cells. Both animal and plant cells are surrounded by a plasma membrane and largely share the same organelles. However, the plant cell is characterized by:

- The presence of a rigid cell wall
- The presence of plastids
- A large vacuole that can occupy most of the cell's volume

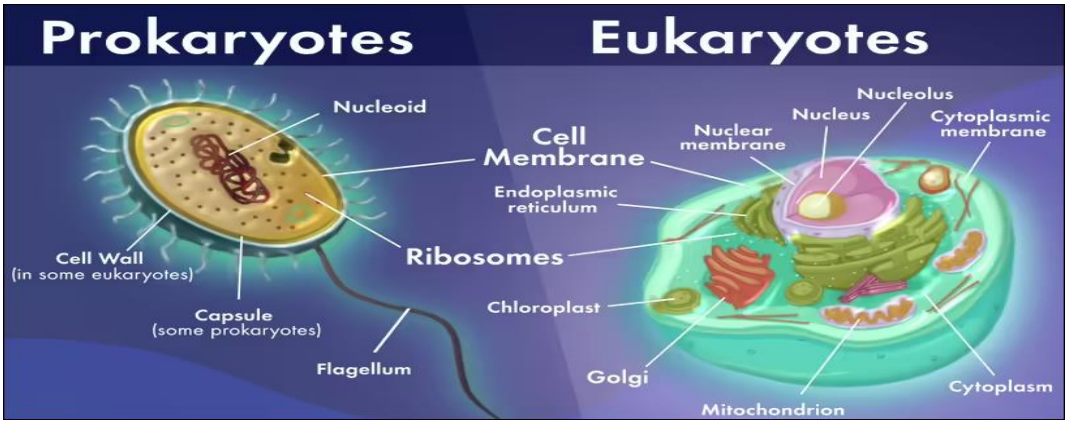


Figure 16: Prokaryotic cells are simpler and lack the eukaryote's membrane-bound organelles and nucleus, which encapsulate the cell's DNA.

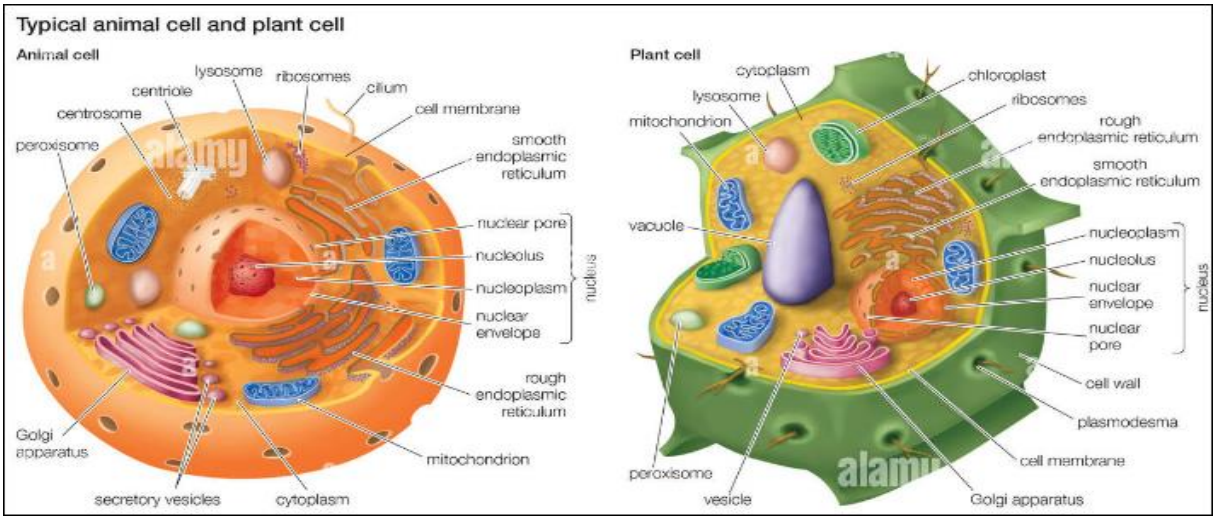


Figure 17: Animal and plant cell

1.2- Chemical composition of the cell

Living beings are made up of three types of matter, which can be observed successively when a living sample is exposed to heat (Table 3):

- **Water vapor** is released first, indicating the presence of water. Its content generally exceeds 60%.
- **The sample turns black** due to the combustion of carbon-rich matter: organic matter.
- **At the end of combustion**, ashes composed of mineral elements remain.

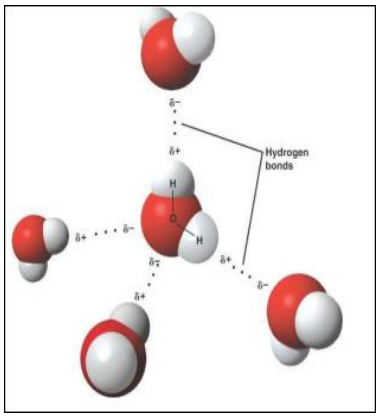
Table 3: Chemical composition of the cell

	PERCENT OF TOTAL CELL WEIGHT	NUMBER OF TYPES OF EACH MOLECULE
Water	70	1
Inorganic ions	1	20
Sugars and precursors	1	250
Amino acids and precursors	0.4	100
Nucleotides and precursors	0.4	100
Fatty acids and precursors	1	50
Other small molecules	0.2	~300
Macromolecules (proteins, nucleic acids, and polysaccharides)	26	~3000

1.2.1- Water in the cell

Water is the most abundant molecule in living cells, accounting for more than 60% of living matter. Its most important roles are:

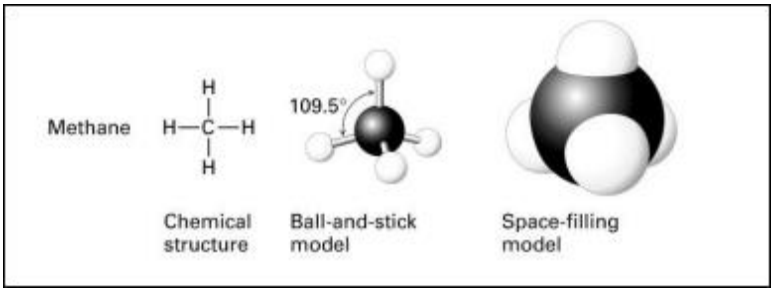
- It is the solvent for many cellular substances.
- It provides the medium in which nearly all biological reactions occur.
- It can participate as a substrate (reactant) or be released as a product in certain reactions, such as hydrolysis.
- Through its interaction with hydrophilic and/or hydrophobic molecules, it helps stabilize many cellular structures, such as membranes.



1.2.2- Organic substances

These are molecules whose main atom is carbon. The simplest molecule is methane, which consists of one carbon atom bonded to four hydrogen atoms. There are four types of organic molecules:

- **Carbohydrates**
- **Lipids**
- **Proteins**



1.2.3- Mineral salts

Mineral salts are the components that remain (as ashes) after the calcination of organic tissues. Chemically, they are ionized elements carrying either a positive charge (cations) or a negative charge (anions).

Mineral salts are essential to the body, notably because they:

- Control water balance (osmotic pressure)
- Regulate acid-base balance (pH)
- Are part of certain structures (bones, teeth)
- Participate in the composition of enzymes and hormones
- Catalyze numerous metabolic reactions

Depending on the quantities involved in the body, mineral salts are commonly divided into two groups:

- **Major elements or macroelements:** Ca, P, K, Cl, Na, Mg
- **Trace elements or microelements:** Fe, Zn, Cu, Mn, I, Mo, etc.

1.2- Study preparations: Sectioning techniques

For a morphological examination of cells or biological samples (specimens) under a microscope, preparatory procedures for the sample are necessary and vary depending on the objectives of the examination.

1.2.1- Whole cells or cell sections in light microscopy

1.2.1.1- Whole cell: Examination of living cells

Living cells can be observed between a slide and a coverslip to assess certain of their functions (for example, motility, measuring the beating frequency of ciliated cells, chemotaxis of neutrophil granulocytes). They can be examined without preparation, but only in very limited cases. This is possible only for isolated cells, either naturally or in culture. An animal cell lacking an exoskeleton dehydrates very quickly in air, so observation must be carried out in a liquid medium. Observation in a culture medium allows the maintenance of cellular physiology.

To properly observe living cells, techniques that enhance contrast without being toxic to the cell must be used.

How to properly observe living cells?

Techniques that enhance contrast without being toxic to the cell must be used.

1.2.1.1.a- Chemical methods (vital dyes)

Almost all dyes are very toxic to cells; only a few rare dyes do not have this drawback. They increase the absorption contrast of certain wavelengths, giving color to the structures that retain them. Example: Janus Green B, specific for mitochondria.

12.1.1.b- Physical methods (phase-contrast microscopy)

This microscope increases the contrast of objects. It is the only way to observe and record cellular movements.

1.2.1.2- Cell sections: Examination of dead cells

1.2.1.2.1- Histology and Cytology techniques

It is necessary to carry out essential manipulations to obtain thin, contrasted specimens. The examination of dead cells, also called histology and cytology techniques, is applicable for both light microscopy and electron microscopy.

Histological processing includes various methods and techniques aimed at studying the molecular and morphological characteristics of tissues. Protocols vary depending on the study's objective and the type of microscope used. For light microscopy, tissues are generally fixed, embedded in paraffin, and then stained to highlight cellular and tissue structures. For electron microscopy, the preparation is more stringent: samples are fixed with specific agents, dehydrated, embedded in resin, and often contrasted with heavy metals to reveal ultrastructural details. These methods can be adapted and combined to achieve the most appropriate results according to the experimental goals (Figure 18, 19).

The technique follows these steps: **sampling, fixation, embedding, sectioning, and staining**.

a) Sampling

In the medical field, samples are taken in clinics, hospitals, or private practices by a specialist physician. Several types of sampling can be distinguished:

- **For cytology:** smear, brushing, puncture
- **For histology:** biopsy, endoscopy, excision

b) Fixation

Fixation is the process of killing cells in a way that preserves structures as close as possible to their living state. Proper fixation must avoid artifacts: the appearance of a new structure (by coagulation), disappearance of a normally present structure (by solubilization), or deformation/displacement of cellular components (by crystallization). Fixation can be achieved by chemical methods, such as alcohol, formalin, acetic acid, etc., or by physical methods, like rapid freezing (the best fixative).

c) Dehydration

To dehydrate tissues, they are immersed in alcohols of increasing concentrations 70°, 80°, 90°, 100° for the time necessary to achieve concentration equilibrium. Paraffin is not miscible with water, so the specimen must be completely dehydrated before embedding in paraffin. Paraffin is also not soluble in the alcohol used for dehydration, so a double substitution is performed:

- Water is replaced by alcohol (**dehydration**)
- Alcohol is replaced by toluene (**substitution**)

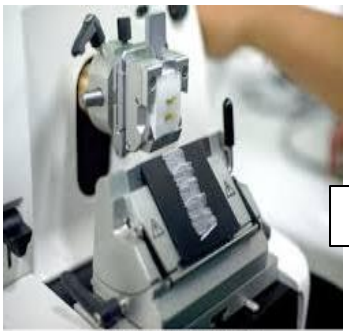
d) Embedding

Sectioning can only be performed in a sufficiently hard substance; therefore, tissues are impregnated in an embedding medium, usually paraffin. Since alcohol is not perfectly miscible with paraffin, the dehydrated tissue is first immersed in an intermediate organic solvent that is miscible with both alcohol and paraffin (xylene), then in paraffin kept liquid in an oven at 50-60°C. The block is then cooled, resulting in a hardened paraffin block containing the tissue to be examined.

This impregnation requires prior dehydration and substitution of water by alcohol, which is the solvent for paraffin.

e) Sectioning

Sections are made using a microtome, producing slices (histological sections) 2 to 5 µm thick. Sections are collected on glass slides. An ultramicrotome is a highly precise device that allows sections 60 to 100 nm thick for electron microscopy.



Microtome

Ultramicrotome

f) Rehydration

Sections mounted on glass slides are deparaffinized using an organic solvent and then brought back to water through baths of decreasing alcohol concentrations.

g) Staining

- Staining for light microscopy:

Cut biological specimens examined by transparency are little or not colored; they provide very low contrast, making visibility poor and details impossible to perceive. The color contrast of different organelles must be enhanced, or the structures must be stained. The glass slide is then immersed in a dye.

Many natural dyes are available, for example:

- **Methyl green:** stains chromatin green
- **Hematoxylin:** stains cell nuclei bluish-purple
- **Eosin:** stains cytoplasm pink
- **Blues** (Methylene blue, Toluidine blue) are also routinely used

- **For electron microscopy:** Staining is actually impregnation (only black and white are visible in electron microscopy) with heavy metal salts, such as lead or uranyl salts, which increase the contrast of cellular structures.

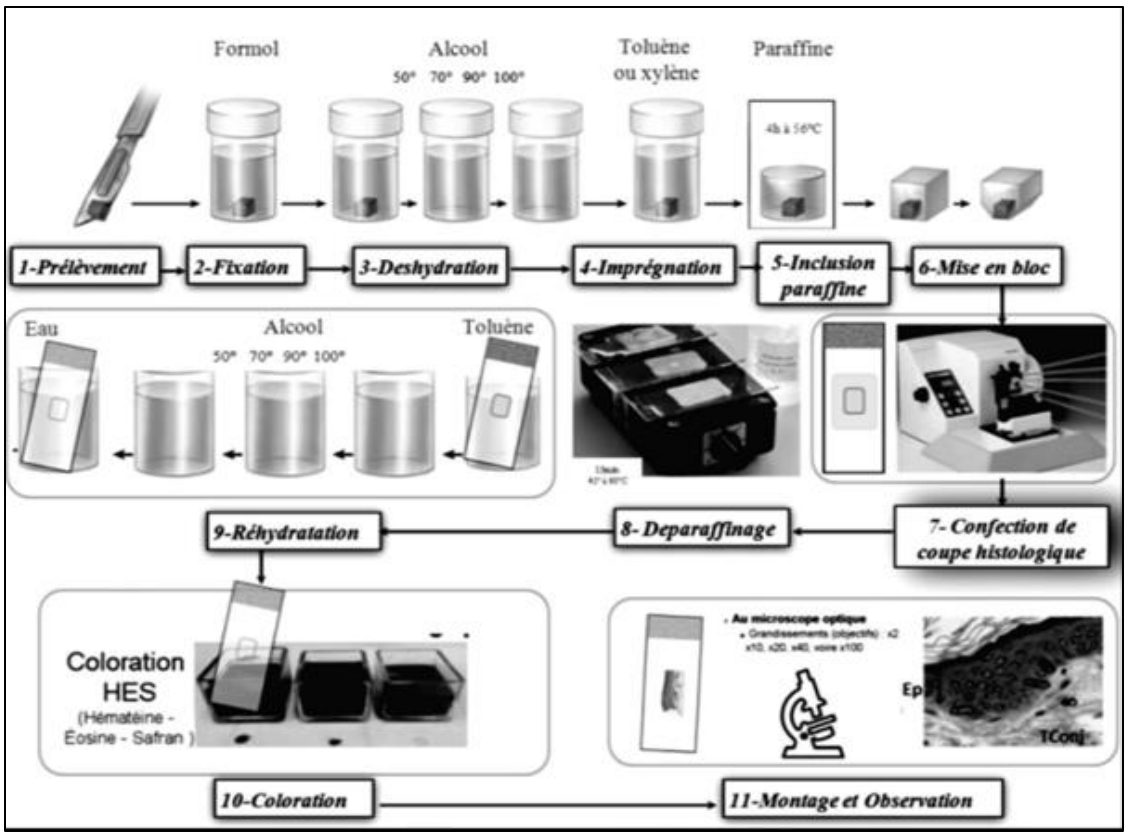


Figure 18: Histological Section Preparation Technique for Light Microscopy

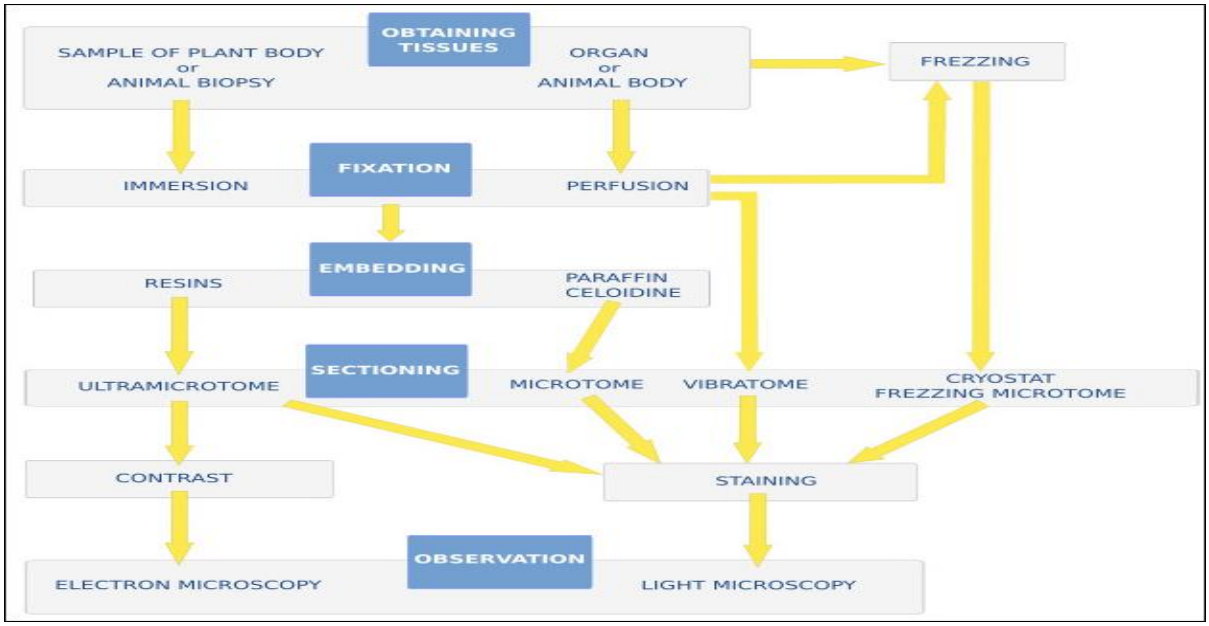


Figure 19: Histological section preparation technique for light microscopy and electron microscopy

- Staining for electron microscopy:

The most commonly used double staining in electron microscopy combines 1% uranyl acetate with alkaline lead citrate. This technique produces the classic black-and-white images of most ultrastructures. To minimize the adverse effects of osmotic variations on proteins, it is preferable to use cryofixation.

Contrasts are achieved by combining high atomic number elements with certain biochemical components of the ultrastructures. Staining, performed before observation, involves immersing the grids carrying the sections for a few minutes in solutions such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid**.**

As the solution evaporates, the metal concentrates and precipitates, forming a layer along the grid except where the sample occupies space. This results in a negative image, created by the contrast between the metal distribution and the emitted electrons (Bouttcher, 2012).

1.2.1.2.2- Replica Techniques: Cryo-etching or Cryofracture

This method produces a topographic imprint (replica) after fracturing a frozen sample. It allows for either a single or double imprint (corresponding to both faces of the fracture). The sample is first fractured after low-temperature fixation to preserve surface relief caused by the heterogeneity of cellular structures. These reliefs are then enhanced by shadowing at a low angle through deposition of a thin metallic layer. The resulting replica can then be analyzed using an electron microscope.

This technique is generally used in scanning electron microscopy (SEM) and proceeds as follows: freezing the sample, cryofracture, surface removal (etching), shadowing, and replica creation.

This method is particularly suited for studying the internal structure of membranes. The replica obtained by cryofracture is already valuable on its own, but its utility can be enhanced by an additional cryo-etching step.

During this phase, the frozen, fractured sample is kept in a cold chamber and placed under vacuum at elevated temperature for a brief period, from a few seconds to a few minutes. This process causes sublimation of a thin superficial ice layer. Once the water is removed, the exposed surface can be coated with a thin layer of heavy metal, allowing detailed structural analysis.

Rapidly frozen tissues help prevent damage caused by ice crystal formation. To achieve this, substances such as glycerol are used, or samples are immersed in extremely cold liquids (such as liquid freon) at temperatures down to -150°C, or directly in liquid helium. Once frozen, the sample is fractured, as illustrated in an explanatory diagram.

Tissue fragments are placed on a small metal disc, frozen, and then fractured by mechanical impact. This procedure highlights the details of the structures traversed, revealing the arrangement of organelles and the composition of certain cells. Shadowing is added to enhance contours and observable reliefs (Figure 20).

The final step involves creating a replica by coating the fractured surface with a thin layer of heavy metal, often platinum or carbon, using a vacuum evaporator. This metallic replica, which preserves the details of the original surface, can then be examined under an electron microscope to analyze the structures in three dimensions (Figure 21-22).

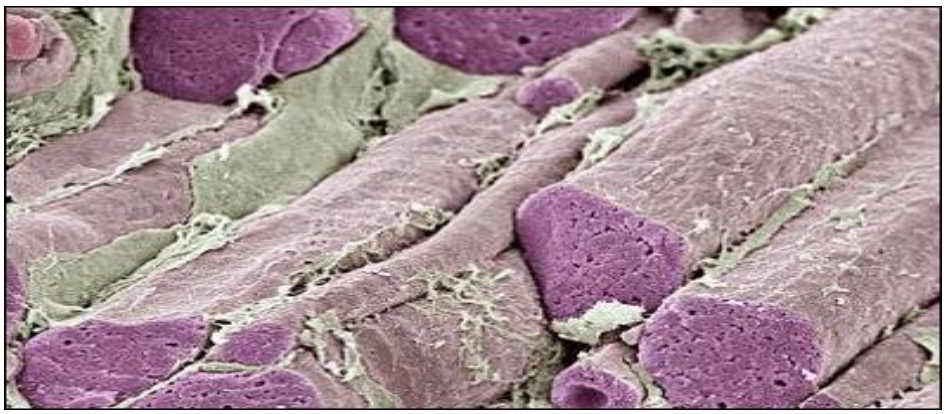


Figure 20: Skeletal muscle fibers, colored scanning electron micrograph (SEM) - Photos 42,43 x 32,03cm

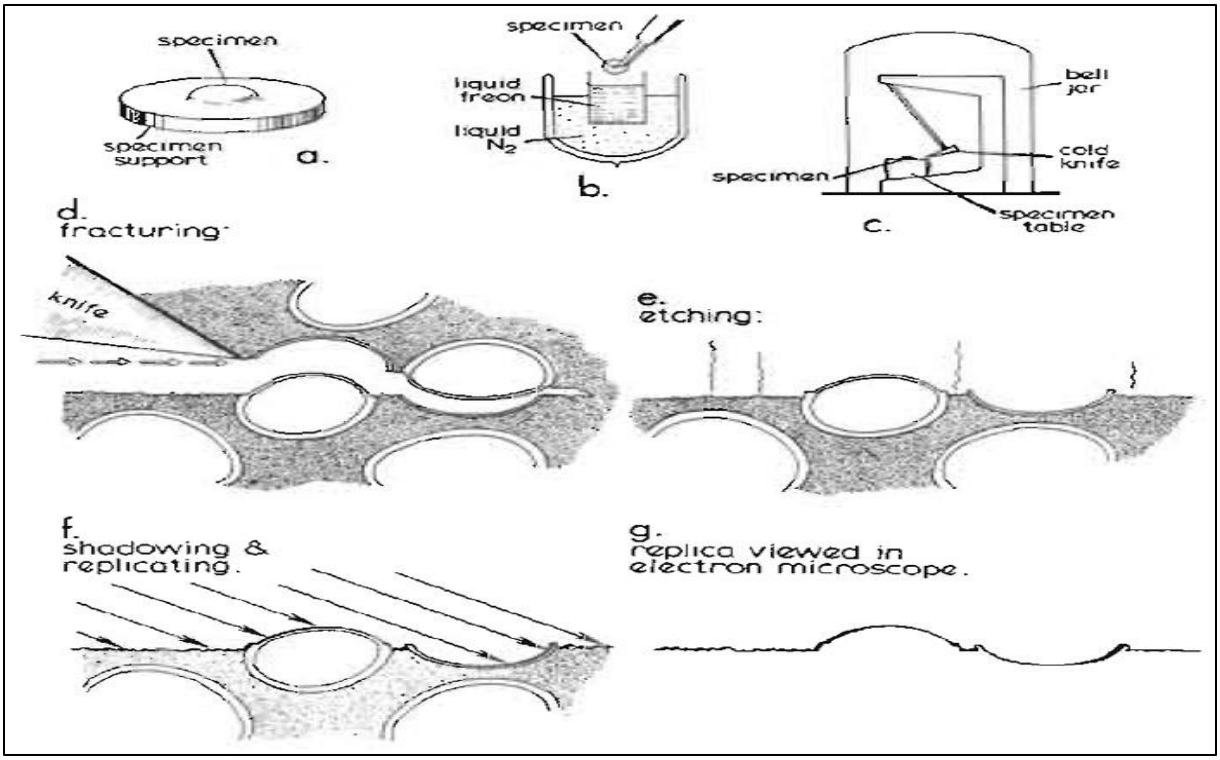


Figure 21: Basic steps in the conventional freeze-fracture procedure described in the text. Diagram of Daniel Branton, first published by Shotton (1982), reproduced by permission

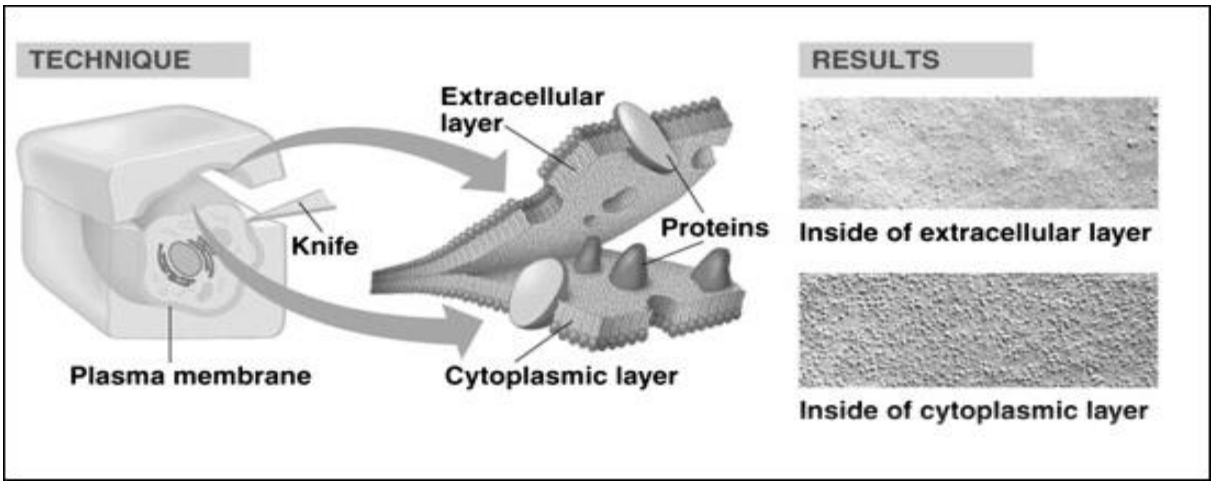


Figure 22: The technique of freeze fracture electron microscopy (Stillwell, 2013)

1.3- Separation of different organelles: Cell fractionation

This is a technique that allows the isolation of cellular organelles while preserving their structure and physiological properties. The organelles obtained by this technique are alive and functional. Their chemical composition can be analyzed, and their function can be studied **in vitro** in a synthetic medium. This technique involves two steps:

- **Cell disruption or preparation of a cell homogenate**, which dissociates the organelles and suspends them in an appropriate medium.
- **Separation of organelles** or homogenization into pure fractions by centrifugation.

1.3.1-Cell disruption or cell homogenate

To obtain a cell homogenate, one can start either from a suspension of cells or from tissue fragments.

Cell homogenate = organelles in suspension + cellular debris (fragments of ultrastructures) + biochemical components in solution.

Cellular tissue is disrupted under the following conditions:

- In an isotonic sucrose solution relative to the intracellular medium to prevent water exchange.
- In a solution with constant pH to prevent proton exchange.
- At 0°C to stop enzymatic activity.

Different techniques can be used:

- Mechanical techniques
- Chemical and enzymatic techniques
- Physical techniques

1.3.1.1– Mechanical techniques

Mechanical grinders are used to reduce the particle size of different types of materials. They are used when the material is in solid, dry, or frozen form.

1.3.1.2-Chemical and Enzymatic techniques

These techniques include lysis or osmotic shock, modification of ionic strength or pH, enzymatic lysis, and the use of detergents.

Enzymatic lysis consists of lysing fragile cells in a hypo-osmotic solution, which first causes swelling (turgor), followed by rupture of the plasma membranes (Figure 23).

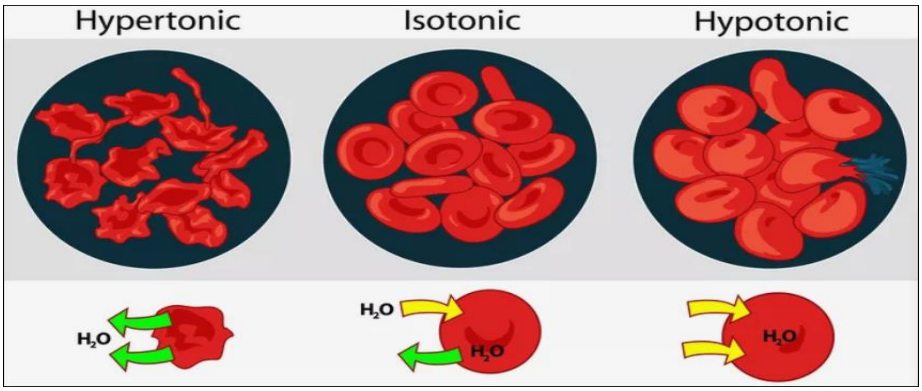


Figure 23: Osmotic pressure and tonicity (Helmenstine, 2025)

The use of lytic enzymes is not a method in itself, but rather a preliminary step that is highly effective for removing extracellular matrices or cell walls.

- *Example 1:* Trypsin and collagenase are used to harvest hepatocytes.
- *Example 2:* Cellulase is used to destroy the plant cell wall.

The use of detergents consists of destabilizing membranes, solubilizing membrane proteins, and forming lipid aggregates in an aqueous solvent.

1.3.1.3-Physical techniques (Figure 24)

13.13.1-Pressure

This technique involves applying pressure to cells, forcing the cell suspension to pass through openings or pores with diameters smaller than that of the cells, resulting in their destruction by rupture of their membranes.

1.3.1.3.2-Sonication or ultrasound

This method is based on the use of a sonicator to destroy cells by producing ultrasonic pressure waves. Since the sonicator generates a significant amount of heat, it is essential to perform the operation under cooling conditions.

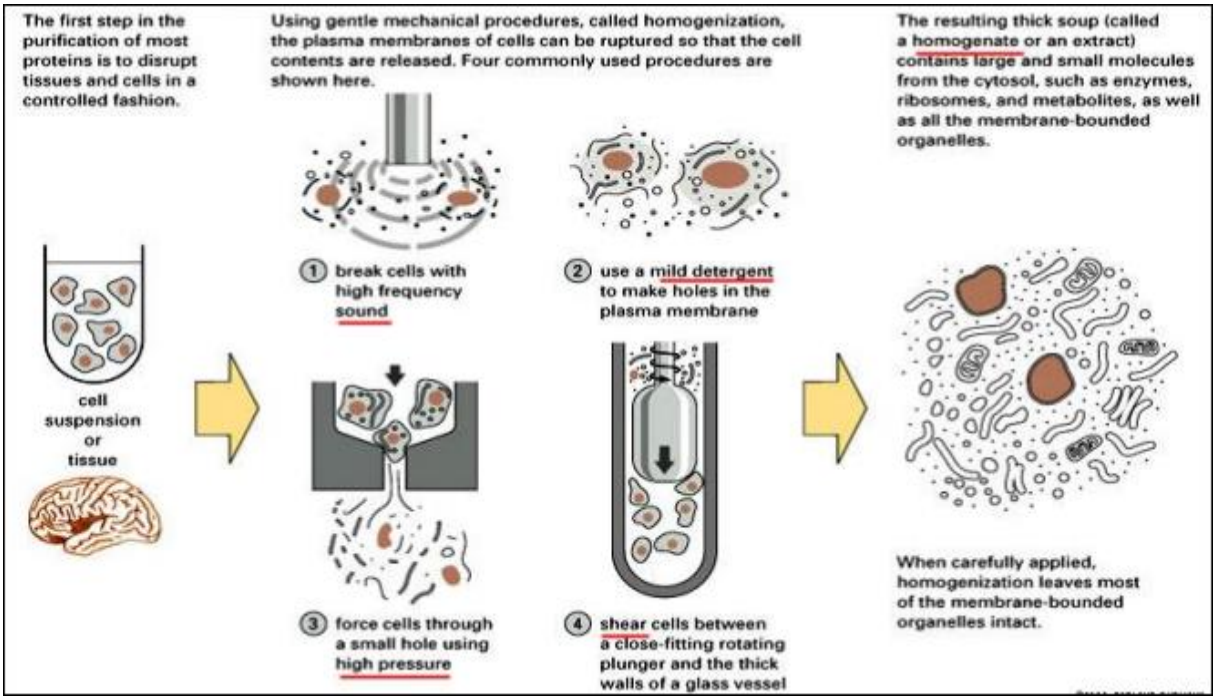


Figure 24: Physical techniques: Breaking cells and tissus

1.4-Separation of organelles or Homogenization (Cell Fractionation)

This refers to the isolation of cellular organelles from a homogenate. If one wants to determine the biochemical composition of cellular organelles, they must be isolated from one another. Organelle purification involves obtaining cellular fractions. This process is referred to as cell fractionation, meaning the isolation of cellular organelles. Cell fractionation is performed starting from a cellular homogenate.

1.4.1- Principle of cellular organelle separation

Cell fractionation is based on differences in organelle density. From a cellular homogenate, organelles can be allowed to sediment, and it can be observed that their sedimentation rates differ because their densities are different:

- The densest organelles sediment first.
- The less dense organelles sediment afterward.

Since the density differences between cellular organelles are very small, separation by gravity alone would take a long time. Therefore, centrifugation is used to replace gravity with a centrifugal force. The acceleration of particles under Earth's gravitational attraction (g) is replaced by a much higher acceleration.

1.4.1.1-Centrifugation

Centrifugation is a technique that allows the separation of components in a mixture according to their density under the action of a centrifugal force. It enables the recovery of a pellet (sediment) and a supernatant. The mixture to be separated may consist of two liquid phases or solid particles suspended in a liquid (Ahern & Rajagopal, 2000).

1.4.1.2-Ultracentrifugation

There are two main types:

1.4.1.2.1-Differential Ultracentrifugation

It is called *differential ultracentrifugation* because it is based on differences in the sedimentation rates of organelles, which themselves depend on differences in organelle density. The term *ultra* refers to the very small differences involved (as in ultrastructure) and to the use of very high accelerations.

Differential centrifugation involves the separation of EVs and other components using a stepwise process. The collected sample is first centrifuged at 300 × *g* for 10 min at 4°C to pellet dead cells and debris. The remaining supernatant is centrifuged at 2000 × g for 10 min at 4°C

to pellet ABs (1000–5000 nm), and the remaining supernatant can be further centrifuged at 10,000 × *g* for 10 min at 4°C to pellet MVs (100–1000 nm). Finally, to isolate the smallest category of EVs, Exos (30–140 nm), the remaining supernatant is centrifuged at 100,000 × *g* for 70 min at 4°C (Lee et al. 2018). Each EV pellet is resuspended in phosphate-buffered saline (PBS) for further analysis or application.

Differential centrifugation is currently the most common method for EV isolation; however, issues arise from the size overlap between the three categories of EVs. For this reason, the ISEV recently changed their naming criteria for EVs because of the difficulty in isolating these distinct populations. This method of EV isolation is suitable for sample volumes, ranging from 1.5 mL to 25 mL (McKenzie et al, 2016), which makes this method ideal for application to human samples. In the isolation of EVs from bronchoalveolar lavage fluid (BALF), differential centrifugation offers consistent results (Lynch et al.2017; Guarnieri et al.2008; Lee et al. 2016).

Example: Isolation of cellular organelles. First, during an initial centrifugation, the heaviest components are isolated. Then, by increasing the sedimentation speed, components with progressively higher densities are separated (Figure 25).

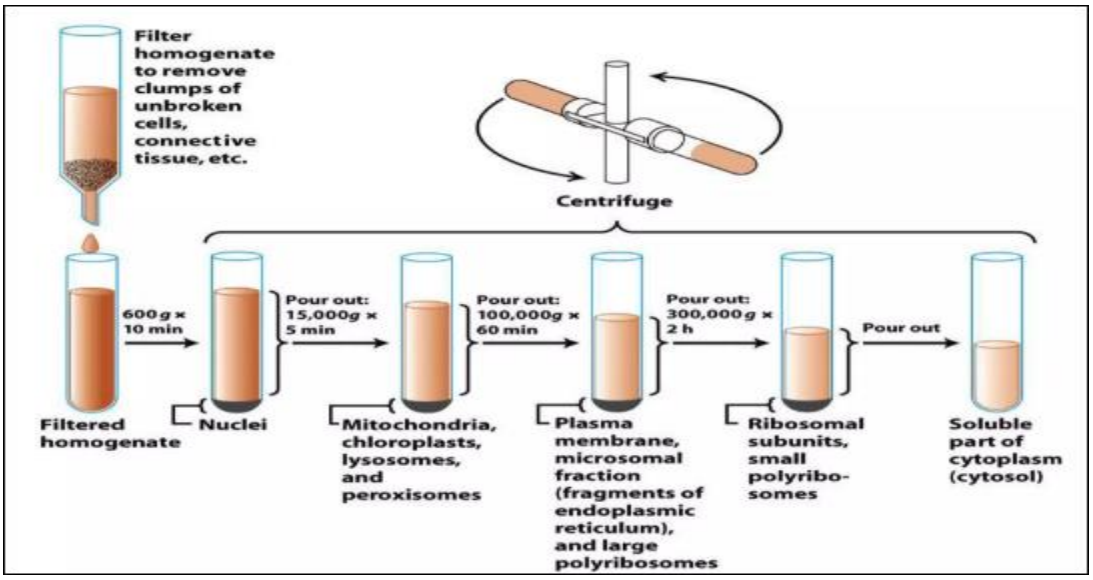


Figure 25: Different stages of differential ultracentrifugation (Molecular cell biology sixth edition 2008)

A medical centrifuge is designed to separate blood components and directly exploits the effect of centrifugal force. Blood is poured into a test tube, which is placed near the center of the centrifuge. When the centrifuge is in motion, the heavier components settle at the bottom of the

tube, while the lighter ones remain at the top. The liquid portion of blood in which the various cells are suspended is called plasma. In the laboratory, plasma can be separated from the cellular components of blood by sedimentation or centrifugation. Both operations result in the formation of a pellet of red blood cells (lower part of the right-hand tube), above which floats a yellowish liquid, the plasma, which contains a large number of proteins. White blood cells and platelets are located between the pellet and the supernatant.

The term ultracentrifugation is used when the acceleration exceeds 20,000 × g (when the acceleration is ≤ 20,000 × g, the process is referred to as centrifugation).

1.4.1.2.2-Density gradient ultracentrifugation

This technique allows the separation, in a single step, of organelles that have very similar sedimentation rates (i.e., very small density differences). It is performed in a medium presenting a density gradient, hence the name of the technique.

The mixture (sample) is deposited on the surface of a preformed sucrose gradient. The molecules migrate at speeds proportional to their size, forming well-defined bands. Once the components are separated, the gradient fractions are collected for further analysis through a small hole made with a needle at the bottom of the tube. The sample must be centrifuged just long enough to separate the target molecules into distinct zones. If centrifuged for too short a time, the molecules will not be sufficiently separated; if centrifuged longer than necessary, all the molecules will eventually form a pellet at the bottom of the tube (Figure 26).

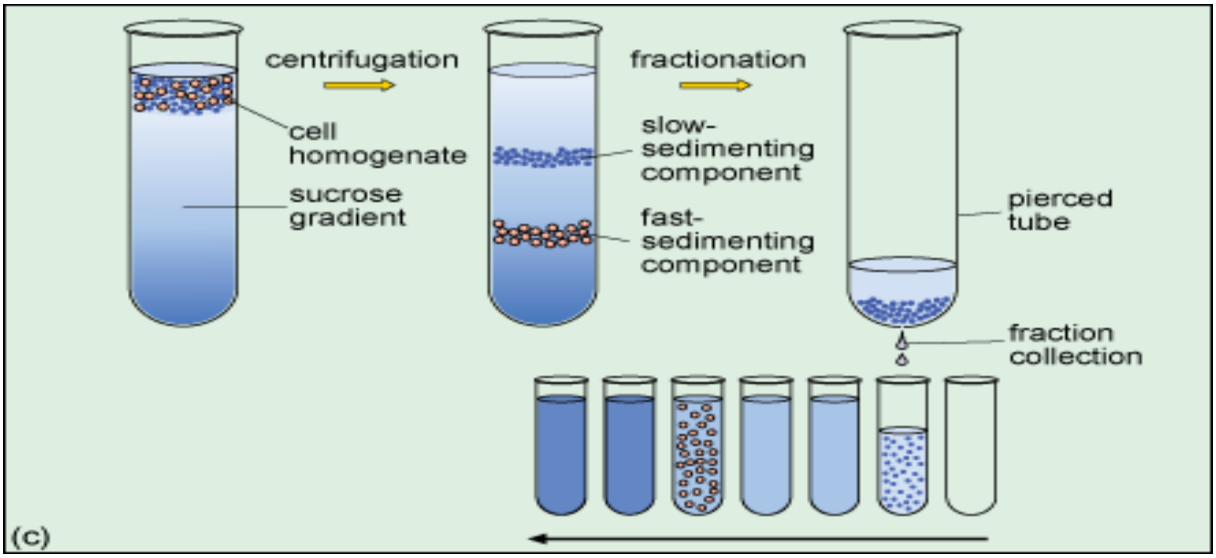


Figure 26: Steps of density gradient ultracentrifugation

2-Biochemical analysis and quantification methods

These methods consist of determining the nature and concentration of biochemical constituents: carbohydrates, lipids, proteins, and nucleic acids.

They are applied either to a mixture (homogenates) or to cellular fractions from which crude extracts are obtained = that is, biochemical constituents in solution. These methods are numerous and include techniques for extraction, purification, characterization, and quantification. As an example, we can consider protein extraction, purification, and quantification techniques.

2.1-Protein extraction

The extraction of a protein from a tissue begins with the disruption of cellular organization (grinding) using mechanical or chemical methods, or through the action of enzymes that disorganize tissues. The mixture resulting from the disrupted biological material and the solvent is called the crude extract **or** homogenate. Cellular debris is removed by centrifugation: the soluble material is collected and washed to eliminate small molecules.

2.2-Purification

Various methods are then used to purify a specific protein from the mixture. In chemistry, purification refers to the separation of chemical substances in order to remove contaminants. There are several purification methods:

- **Filtration:** Based on differences in the size (diameter) of solid particles dispersed in a liquid.
- **Centrifugation:** Based on differences in density.
- **Electrophoresis:** Based on differences in charge.
- **Chromatography:** Based on differences in solubility.
- **Spectrophotometry:** Used for detection and quantification.

2.2.1-Electrophoresis

2.2.1.1-Introduction

Electrophoresis is an analytical technique based on the differential migration of ionized molecules subjected to a continuous electric field, within a buffered electrolytic medium of defined pH and ionic strength. The origin of this method was first proposed by S.E. Linder and H. Picton in 1892.

2.2.2.1.2-Principle

The electrophoresis technique is based on the movement of ions (positively or negatively charged molecules) under the influence of an electric field. Due to their intrinsic characteristics and depending on electrophoresis conditions, these ions migrate at different speeds, allowing them to separate from one another.

Depending on the properties of the molecules and the electrophoresis conditions, the migration rate varies between charged molecules, enabling their separation.

* Cation: positively charged; attracted during electrolysis toward the cathode (negative electrode).

* Anion: negatively charged; attracted during electrolysis toward the anode (positive electrode).

This technique has some applications in chemistry, but it is mainly used in biochemistry and molecular biology for the separation of proteins and nucleic acids, including DNA.

2.2.1.3-Different types of electrophoresis

The choice of support depends on the nature of the molecules to be separated. According to the support used, three types of electrophoresis can be distinguished:

1/Free-solution electrophoresis (liquid support):

2/Zone electrophoresis (on a porous support):

•On paper or cellulose acetate (solid support),

•On agarose gel or polyacrylamide gel (semi-solid support),

•Isoelectric electrophoresis (isoelectric focusing),

3/Capillary electrophoresis

2.2.1.3.1-Free-solution electrophoresis (liquid support)

An old method (developed by Arne Tiselius in 1937), generally used for the separation of large particles (cells, organelles).

In this type of electrophoresis, migration occurs within a liquid of defined composition subjected to a continuous electric field. It is carried out in a U-shaped tube with a square cross-section (this allows optical measurements through the tube, similar to a spectrophotometer cuvette). The separation is not complete, but the boundaries that form are detected using optical methods (UV absorption, refractive index, fluorescence, etc.) (Figure 27).

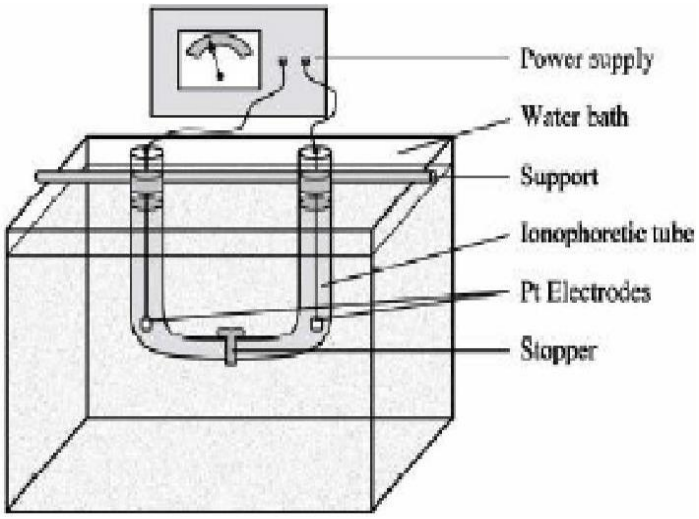


Figure 27: Electrophoresis setup (Singh, 2012)

2.2.1.3.2-Zone electrophoresis (on a porous support)

Molecules migrate mainly according to their overall charge under non-denaturing conditions. This method is used for the separation of small molecules (amino acids or small peptides).

2.2.1.3.2.1- Paper electrophoresis

Has relatively low resolution; this technique is mainly intended for separating small molecules, particularly amino acids. Interference phenomena related to the charges of amino acids and the cellulose of the paper play a significant role. It is therefore useful for the separation of amino acids (Figure 28).

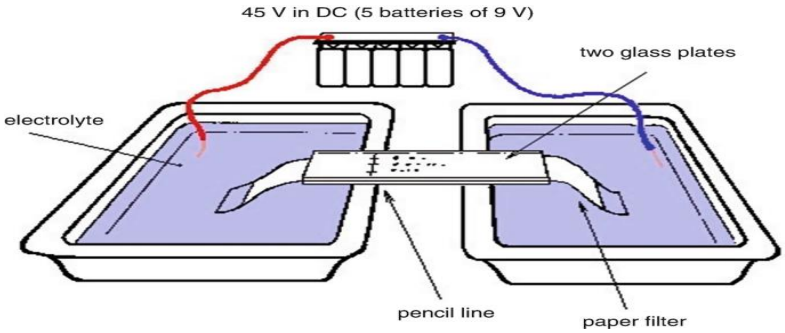


Figure 28: Paper electrophoresis (Basha, 2019)

2.2.1.3.2.2- Cellulose acetate

Is performed under conditions similar to those of paper electrophoresis. Cellulose acetate strips are fragile, but they limit the diffusion of the molecules to be separated. Protein detection is carried out using a colorimetric reaction (for example, Ponceau red staining). This technique has low resolution but allows a rough separation of groups of proteins. It is inexpensive and enables rapid analysis of serum proteins.

2.2.1.3.2.3- Gel electrophoresis

Gel electrophoresis is one of the most widely used and efficient routine methods for separating macromolecules. Commonly used gels (polyacrylamide and agarose) have pores of molecular dimensions whose size can be controlled.

- ✓ **Principle**

The separation of molecules in gel electrophoresis depends on both electrophoretic mobility and gel filtration. The gels used in this technique slow down the migration of large molecules compared to smaller ones. Thus, the larger the protein, the more it is hindered by the gel matrix and the more slowly it migrates. This is the opposite of the principle of size-exclusion chromatography, where there is space for the solvent between the beads. In contrast, in gel electrophoresis, the gel is directly cast within the electrophoresis apparatus.

Among the gels used: Polyacrylamide gel and agarose gel

2.2.1.3.2.3.1-Vertical protein electrophoresis

Relies on the use of an ionic detergent, SDS (sodium dodecyl sulfate). By binding to proteins, SDS denatures the polypeptide chains and imparts a uniform negative charge proportional to their length. Under these conditions, smaller molecules migrate more rapidly through the gel matrix toward the anode, while larger molecules move more slowly. When the smallest molecules reach the end of the gel, all components of the sample are separated according to their size (Pollard et al., 2004) (Figure 29).

Figure 29: Vertical electrophoresis chambers

2.2.1.3.2.3.2- Horizontal electrophoresis of nucleic acids (DNA, RNA) on agarose gel

In the electrophoresis of nucleic acids (DNA or RNA), sample preparation does not require the use of chemical additives such as SDS, since these molecules naturally carry a negative charge. This property causes them to migrate toward the anode under the effect of an electric field, producing a migration pattern (Figure 30).

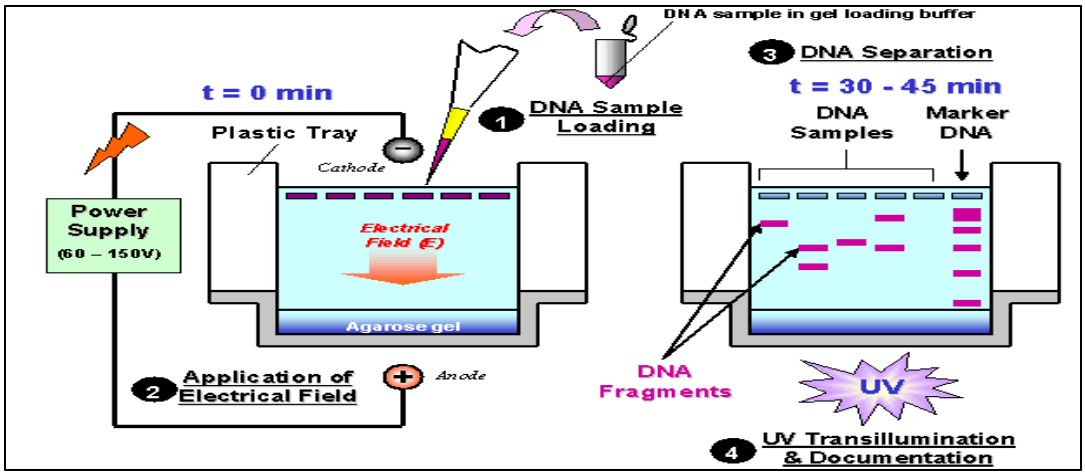


Figure 30: Steps involved in agarose gel electrophoresis

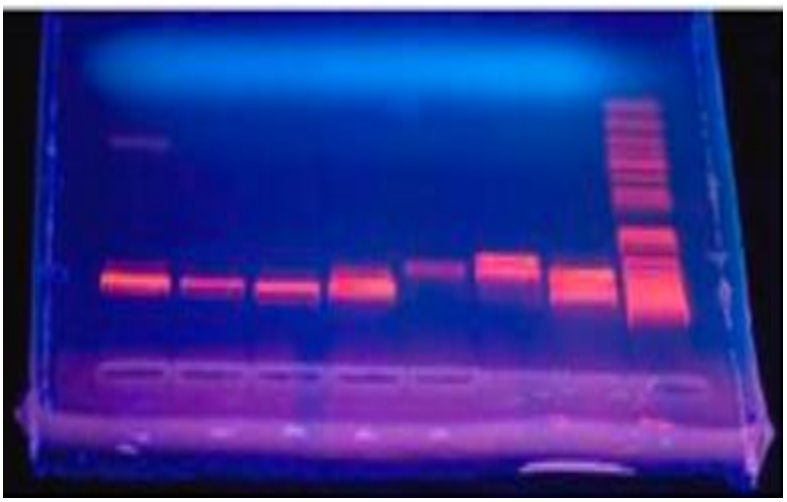


Figure 31: DNA Electrophoresis Under Uv Light. DNA electrophoresis under UV light. Close-up of a DNA (deoxyribonucleic acid) electrophoresis gel viewed under UV (ultraviolet) light. Gel electrophoresis is a technique used to analyse the molecular composition of DNA samples. The extracted DNA is placed into a gel, which then has an electrical current passed through it that separates the DNA molecules based on their size and charge. The technique is also used to analyse the protein composition of cells and tissues (Louise Murray, 2016)

2.2.2-Chromatography

For the identification and quantification of organic matter, chromatographic techniques are generally used.

2.2.2.1-Gas Chromatography

Gas chromatography consists of three main parts: an injector, a separation column, and a detector. After introducing the sample using a microsyringe through the injector, the molecules are separated in the column according to a temperature gradient. As the separated compounds exit the chromatographic column, they pass individually through a detector, whose function is to produce a signal (in the form of a peak) with an intensity proportional to the amount of compound injected.

2.2.2.2-Liquid phase chromatography

2.2.2.2.1 – Column Chromatography (CC)

The active substrate is packed into a tube and can be either an adsorbent (liquid–solid chromatography, LSC) or a support acting by partition (liquid–liquid chromatography, LLC).

In both cases, the mobile phase is a liquid, used either at atmospheric pressure or under pressures reaching several hundred bars. In the latter case, the technique is called high-performance liquid chromatography (HPLC).

2.2.2.2.2- Ion-exchange chromatography

The stationary phase consists of a specific solid called an ion exchanger, carrying fixed functional groups that are ionized or ionizable. The ions maintaining electroneutrality of the structure are mobile and can be exchanged with ions present in the mobile phase in contact with the exchanger, which is generally grafted onto polymers or silica-based supports.

2.2.2.2.3-Gel Permeation Chromatography

The stationary phase is a solid, most often made of silica or alumina, filling a column. The sample is applied at the top of the column. Separation of chemical molecules occurs via the continuous flow of a mobile phase, or eluent, through the column. This process relies on differences in migration rates of substances down the column, which depend both on each molecule's adsorption capacity by the stationary phase and the driving force applied by the eluent.

2.2.2.2.4-Paper Chromatography (PC)

The substrate consists of the charge (water, salts, etc.) and fibers of a suitably chosen paper, with the solvent moving through the paper by capillary forces.

2.2.2.2.5 -Thin-Layer Chromatography (TLC)

Follows the same principle, but the substrate is spread and fixed on an inert plate, such as glass or another material.

2.2.3-Spectrophotometry

2.2.3.1-Molecular absorption spectrophotometry

This technique is based on the fact that any colored solution, when traversed by a beam of light, allows only a fraction of the incident light to pass through. The amount of light

absorbed is proportional to the concentration of the colored compound being measured (Beer-Lambert law).

2.2.3.2-Atomic absorption spectrophotometry (AA)

This so-called elemental analysis method allows the quantification of chemical elements present in trace amounts (very low concentrations, e.g., a few ppm) in a solution.

Chapter III. Cytochemical and Immunological Methods

1-Cytochemical methods

These methods allow the localization of biochemical constituents within cells. They are applied to histological sections. Several examples are given for illustration.

1.1-Autoradiography

Autoradiography is a simple and sensitive photochemical technique used to record the spatial distribution of radiolabeled compounds within a specimen or an object. The purpose of autoradiography is to label a specific molecule with radioactivity (Robert & Farrell, 2010). It allows tracking and localizing substances at the level of cellular organelles. This technique relies on the use of radioactive products that have two essential properties (Figure 32):

- They are used by living organisms in exactly the same way as their non-radioactive isotopes (chemical elements identical except for atomic mass).
- They emit radiation that can be detected using a photographic emulsion.

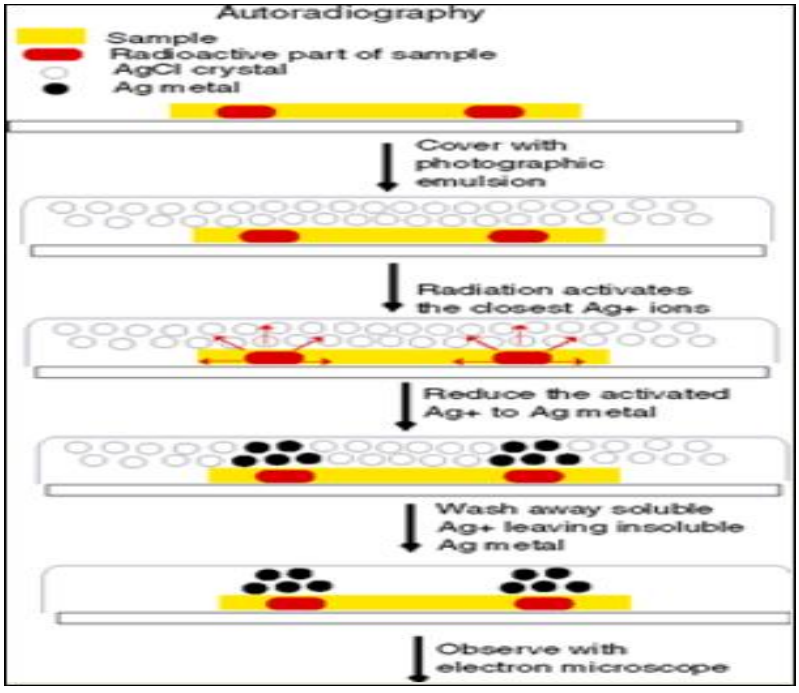


Figure 32: Methdoloogy of autoradiography
http://lifeofplant.blogspot.com/2011/12autoradiography.html

1.2-Immunocytology (Antigen Localization Technique)

Immunocytological techniques are based on the use of an antibody (Ab) coupled with a marker, visible under a light microscope. These methods rely on the specific recognition property of antibodies, meaning that once an antibody is available, it can be used to locate any antigen (Ag). These techniques are always applied to tissue sections.

The method takes place in two steps:

1. **Formation of the Ag-Ab complex = immune complex:**
   The tissue section is immersed in a bath containing the primary antibody in solution.
2. **Detection of the immune complexes:**
   Immunological tools, called anti-antibodies = anti-immunoglobulins = anti-Ig = labeled secondary antibodies, are used.

- There are different ways to label the "detecting antibodies":
  - **With enzymes** capable of producing a colored product → observable by transmission light microscopy.
  - **With fluorochromes** → observable under a fluorescence microscope.
  - **With radioisotopes**
- Depending on the markers used, there are three types of immunological techniques:

1.2.1-Immunofluorescence

Immunofluorescence (IF) is an immunochemical staining technique that utilizes fluorochrome-labeled antibodies for the visualization and localization of targets, by labelling with a fluorescent dye such as fluorescein isothiocyanate (FITC). It can identify a wide range of components on tissue sections, cultured cells, or individual cells, and is thus highly valuable for their broad capability with the use of combinations of specific antibodies tagged with fluorophores (Im et al., 2019).

Example: To detect substance P in a cell, the following steps are followed:

1. A protein P (taken from a mouse) is injected into an animal (e.g., a rabbit). The rabbit reacts by producing **anti-P antibodies**.

2. A fluorochrome molecule (e.g., fluorescein) is directly attached to the antibody, forming a complex (**anti-P antibody–fluorochrome**).
3. After adding the labeled antibodies to the cell preparation, they bind to antigen P, forming the **antigen P–fluorescent antibody complex**.
4. Observation under UV light with a fluorescence microscope allows localization of substance P, thanks to the fluorescence emitted by fluorescein.

- Immunofluorescence can be **direct** or **indirect**, depending on the method of labeling and detection (Figure 33).

- Direct method: In this method, the target molecule is directly coupled to a labeled antibody.

- Indirect method: In this method, the marker is coupled to a second antibody, or secondary antibody, which is specific to the immunoglobulins of the species that produced the primaryantibody.

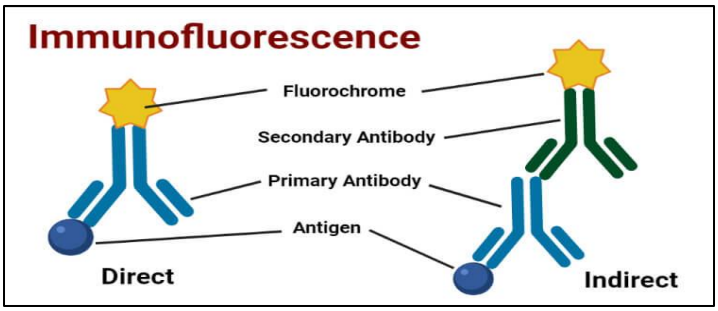


Figure 33: Direct and Indirect immunofluorescence (Dwivedi, 2022)

1.2.2-Radioimmunology

This is a quantitative assay and analysis technique based on the principle of the antigen-antibody reaction combined with a radioactive label (radioisotope).

1.2.3-Immunoenzymology

This method allows the detection of the presence or absence of a protein in cells of a paraffin-embedded tissue section, as well as its localization.

- **Principle:** A protein of interest is identified using a specific primary antibody, which is in turn recognized by a secondary antibody directly coupled to an enzyme or linked to a polymer coupled to an enzyme.
- The enzyme can be horseradish peroxidase (HRP) or alkaline phosphatase (AP).
- The antigen–antibody–enzyme complex is visualized using a specific substrate for HRP or AP.

1.3-Enzymatic methods

1.3.1-Localization of polysaccharides: Periodic Acid–Schiff (PAS) Reaction

This is the most commonly used method to localize polysaccharides in cells. It is performed on tissue sections in two steps:

1. Periodic acid (HIO4) is an oxidizing agent that cleaves 1,2-glycol and amino alcohol linkages in carbohydrates and glycoproteins, forming poly-aldehydes.
2. These aldehydes then react with **Schiff reagent (acid fuchsin)**, producing a purplish-red color in structures containing aldehydes.

1.3.2-Localization of nucleic acids: Brachet Test

This technique is used to localize nucleic acids in cells and is always performed on sections.

- The test combines a staining method using a mixture of methylene green + pyronin red:
  - Methylene green → DNA appears green
  - Pyronin red → RNA appears red
- Specific hydrolytic enzymes (DNase for DNA or RNase for RNA) are applied as needed to confirm the localization (Figure 22).

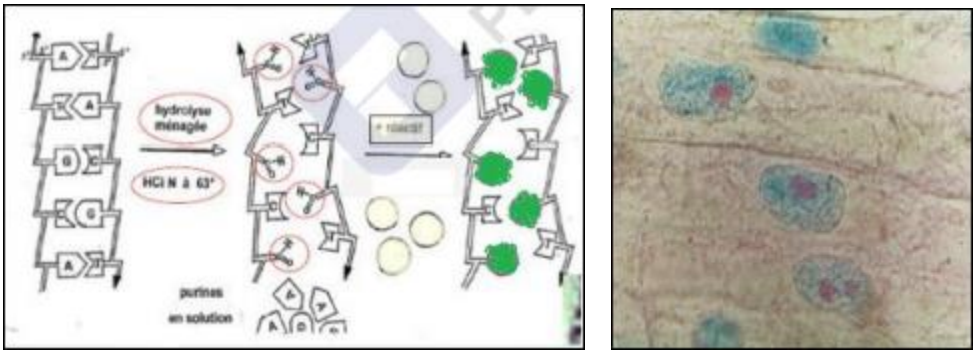


Figure 34: DNA localization reaction

Chapter IV. Genetic Engineering Techniques (DNA Sequencing)

DNA sequencing consists of determining the order of nucleotides in a given DNA fragment.

The method is carried out in several steps:

1-Preparation of nucleic acids (Extraction and Purification)

DNA extraction is a technique that allows the isolation of DNA from cells or tissues. The extracted DNA can then be used for molecular biology research, such as sequencing, PCR, or cloning.

There are different protocols for extracting DNA, but they generally follow the same principle:

- Cell lysis
- Removal of proteins
- Removal of other nucleic acids (RNA, etc.)
- Concentration of DNA by alcohol precipitation

2-*In-Vitro* amplification of nucleic acids: PCR (Polymerase Chain Reaction) and RT-PCR (Reverse Transcription PCR)

2.1-PCR

PCR (Polymerase Chain Reaction) is a biochemical technique developed by Kary Mullis in 1983 that is used to create large quantities of a sequence of DNA. Since this method of mass-producing DNA was first introduced, it has become significantly less labour intensive, more economical, and more routine. The PCR allows in vitro amplification of a specific region of a given nucleic acid to obtain a sufficient quantity for detection and study (Reina, 2025).

For replication of double-stranded DNA**,** the reaction occurs in three steps (Figure 35):

2.1.1-Denaturation: DNA is heated to approximately 95°C to separate the double strands and obtain single-stranded templates.

2.1.2-Annealing and primer hybridization: Specific oligonucleotide primers are used to initiate replication of the sequence to be amplified. This hybridization, which pairs the complementary bases of the primer with the DNA template, is facilitated by DNA ligase and occurs at a temperature defined according to the primer properties (typically 50–60°C).

2.1.3-Extension (Polymerization): The complementary strand is synthesized using DNA polymerase.

At the end of each cycle, the products are double-stranded DNA. Polymerization occurs at approximately 72°C, which is the "working temperature" of the thermostable DNA polymerase used. During this step, complementary DNA strands are synthesized from the free 3'OH ends of the hybridized primers. The number of cycles is determined by the experimenter according to the desired level of amplification. In most cases, this number is approximately 30 cycles, resulting in an amplification factor of around 2^{30}.

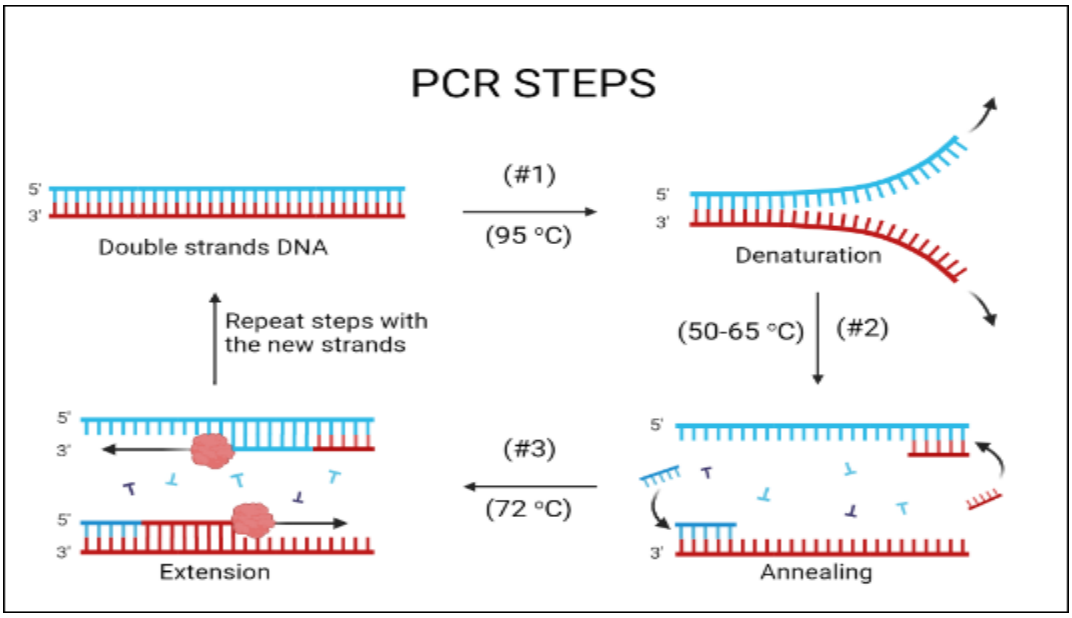


Figure 35: The three-step process of the polymerase chain reaction

2.2-RT-PCR

RT-PCR takes place in two phases:

1-. he first phase corresponds to copying messenger RNA (mRNA) into complementary DNA (cDNA).

2- The second phase is a classic PCR reaction performed on the synthesized cDNA.

3. DNA Sequencing (Sanger)

Sanger sequencing is defined as a method used to determine the nucleotide sequence of a specific region of DNA by synthesizing new strands from short complementary sequences (primers) and analyzing the resulting mixture of DNA strands using electrophoresis to identify the order of nucleotides. This technique is primarily employed in genetic testing to detect mutations in known genes, although it has limitations in assessing longer DNA segments and certain types of genomic alteration.

Currently, DNA sequencing is mostly based on the enzymatic Sanger method.

- This method generates DNA fragments terminated by one of the four bases, which are pre-labeled, because the chain elongation stops after incorporation of a dideoxynucleotide (ddNTP) (Figure 36).
- This is the fundamental principle underlying the method.

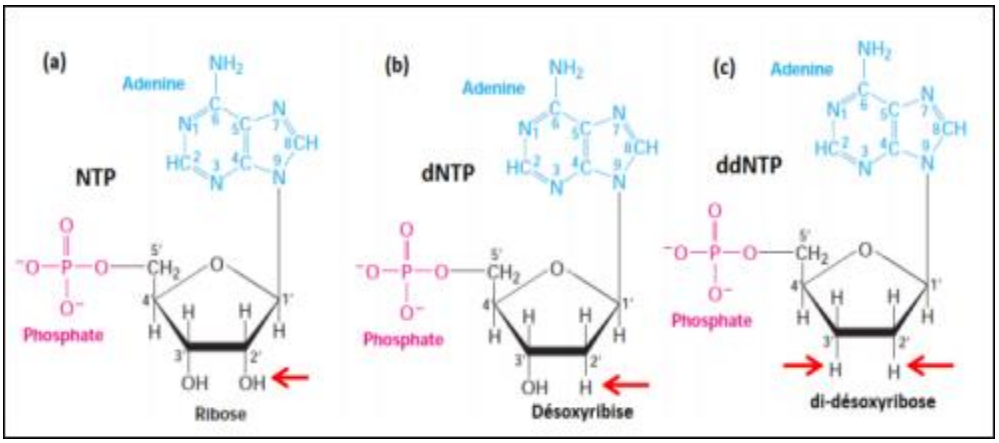


Figure 36: The different forms of DNA nucleotides

In ddATP, the 3'-OH group is replaced by a hydrogen. This modification prevents further DNA synthesis, which normally continues from the 3'-OH.

The Sanger method allows the determination of an unknown DNA sequence using a single-stranded DNA template. This is carried out with DNA polymerase and a specific primer.

To better understand the principle of sequencing, unlabeled ddNTPs are used:

- Four separate tubes are prepared, each containing the four deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) along with a small amount of one of the four dideoxynucleotides (ddATP, ddCTP, ddGTP, or ddTTP), DNA polymerase, primer, nuclease-free water, $MgCl_2$, and the DNA to be sequenced.

These dideoxynucleotides act as chain terminators: once incorporated into the newly ynthesized strand, they prevent further elongation.

3.1-Determining the nucleotide sequence using the Sanger method involves two steps

1. **Synthesis of the complementary strand** from a template strand using DNA polymerase and a primer, in the presence of the four dNTPs and a ddNTP serving as a chain terminator (lacking a 3'-OH). Four parallel reactions are performed, each containing one of the terminators at a low concentration.
2. **Separation of the synthesized fragments by electrophoresis (PAGE):**
    - The number of fragments depends on the number of nucleotide residues.
    - The position of the chain terminators indicates the position of each nucleotide in the sequence.

3.2-Automation of DNA Sequencing

In automated sequencing, fluorescent labels are used, with a different marker for each ddNTP (e.g., blue, red, green, orange, etc.).

- Polymerization is carried out in a single tube containing the reaction mixture and all four ddNTPs.
- The dideoxynucleotides are labeled with different fluorescent dyes, allowing a single reaction and single readout.
- The sequencer detects the fluorescence and determines the size of each DNA fragment.

- This produces fluorescence curves that are interpreted to identify the nucleotide sequence.

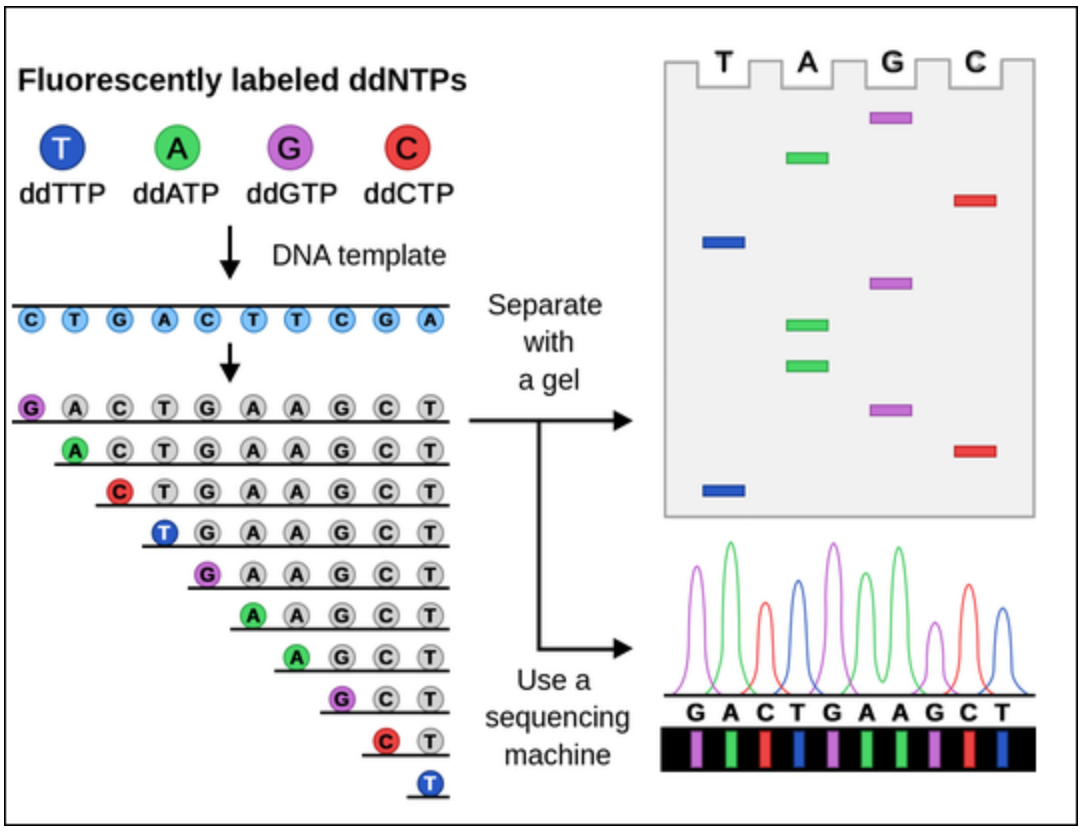


Figure 37: Schematic overview of Sanger (chain-termination) method for DNA sequencing

Part Two: Methods and Techniques for Studying Living Organisms

The experimental approach in biology is based on several essential steps: exploration, observation, manipulation, and experimentation. To better approximate the realities of living systems, biologists rely on various techniques that facilitate their study.

These approaches to studying living organisms encompass a set of methods involving more or less direct contact with organisms. This includes maintaining them under controlled conditions (breeding, culture), studying them in their natural environment (collection), as well as analyzing dead organisms (dissection). In this section, we will examine these different approaches and their main applications in biology.

I.1-Herbarium

1.1-Definition

A herbarium is typically a collection of dried plants preserved primarily for scientific purposes, and sometimes for aesthetic reasons. The earliest herbaria date back to the 16th century, and these collections constitute an essential resource for research in phytotaxonomy and its applications, including geographical distribution of species (chorology), ecology, and plant products**.**

How to make a herbarium?

1.2-Steps for creating a herbarium

1.2.1-Selection and Collection

This initial step is crucial, as it allows you to select the target species directly in the field.

- The specimen should then be cleaned by manually removing dead leaves and any soil attached to the roots.
- Choose plants that are typical representatives of the population.
- Avoid collecting isolated individuals, as this may destroy rare plant populations.

- Do not harvest on rainy days or when there is heavy dew, as the samples may rot during drying.
- Collect the entire plant, including:
  - Root system
  - Basal and stem leaves
  - Reproductive parts (flowers, fruits, cones, or sporangia)
- Some species are protected**,** and their collection is prohibited.

2.2.2-Identification of the sample

- Each specimen is numbered with a label.
- The number, along with all relevant information about the collection site, is recorded in a field notebook.

2.2.3-Storage and Transport

To preserve samples before drying (for identification, for example), a plastic bag can be used, or better, an old directory in which each sample is slipped.

1.2.4-Drying

Plants are placed to dry between the pages of old newspapers or in an old directory, with pressure applied (plant press, weights, books, or stones). Newspapers must be changed frequently (daily at the beginning) to prevent rotting.

Pressed plants must be thoroughly dried prior to storage and mounting. Best results are obtained with steady airflow and bottom heat between 95–120°F. Electric dryers are most often used, but in the field combustible fuel-based heaters may be used (see Blanco et al. 2006 and the Plant Specimen Collection and Pressing Bibliography).

Figure 38: Layout of a plant specimen

1.2.5-Mounting in the herbarium

- Once dried, the plant is mounted in a notebook, binder, or on loose sheets.
- The plant should be spread as flat as possible, with leaves uncrushed if possible.
- The specimen is attached using small pieces of tape.

Figure 39: A "Specimen Voucher" from the US National Herbarium that is a dried, pressed herbarium voucher specimen with Catalog Number and specimen label. The collector + number is Van Neste 340, the sheet number is 3698273 and the specimen barcode is 01246627(doi for all figures 10.6084/m9.figshare.4496978)

1.2.6-Archiving

At the bottom right corner of the page, write the following information as completely as possible:

- Phylum
- Subphylum
- Class
- Family
- Genus and species
- Common names: French, Arabic, other languages
- Date and place of collection
- Type of habitat

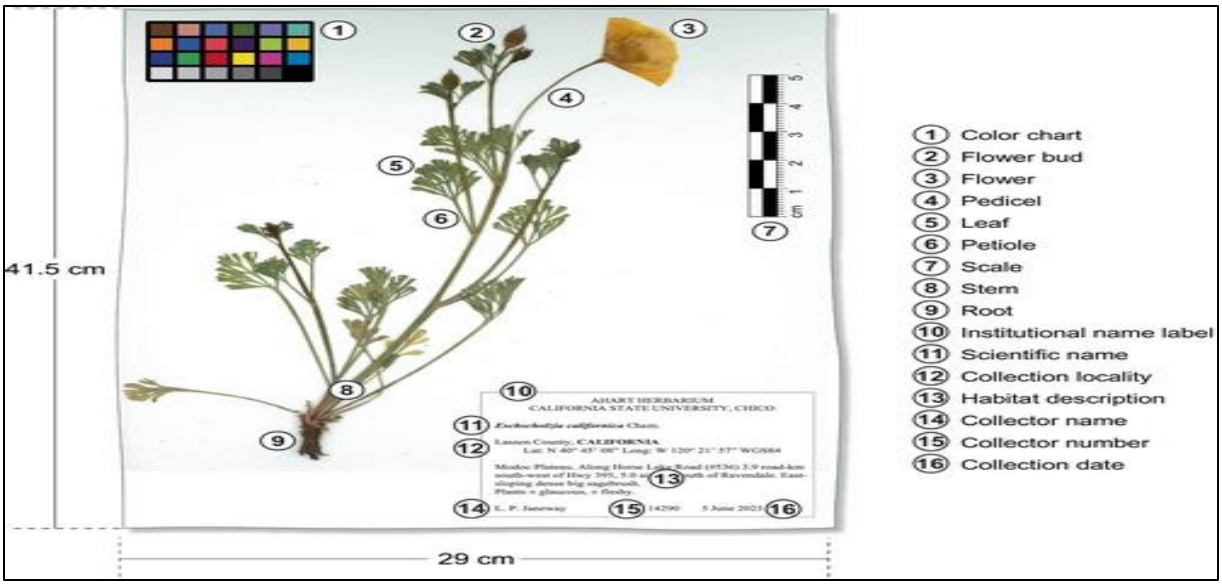


Figure 40: Annotated herbarium sheet of California poppy (Eschscholzia californica). Illustrated are the key components of a herbarium specimen that are relevant to understanding plant biogeography, including the plant's morphological features (e.g., flower, leaf, stem, and root) and associated metadata such as institutional name, scientific name (Eschscholzia californica), collection locality, collection description, collector name (L. P. Janeway), and collection date (5 June 2023). Measurement tools (scale bar and color chart) provide additional context for accurate identification and size measurement of the specimen. Credit: California State University Chico Herbarium (CHSC: CC BY-NC 4.0). This figure was created in Power Point v.16.92 (24120731) (Barnabas H. Daru, 2025).

II.2-Breeding

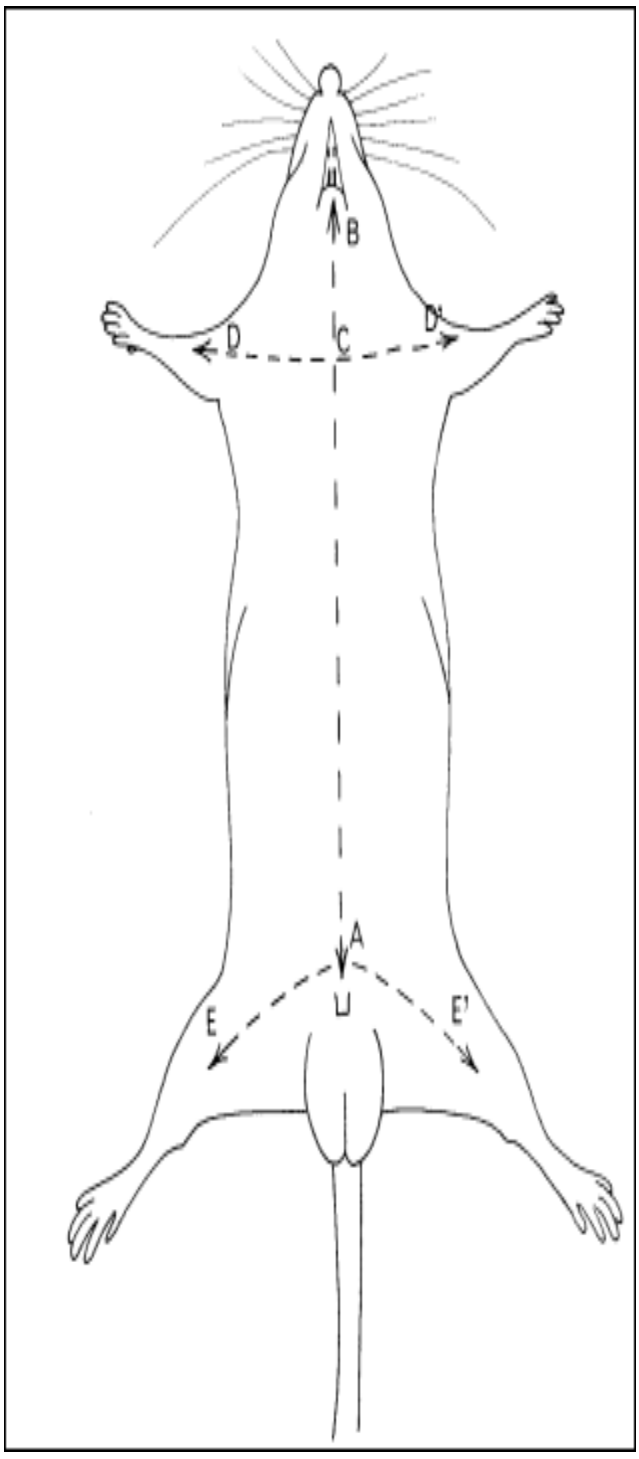
Breeding encompasses all activities related to the care of animals, including providing food, care, and protection, for various purposes such as food production, economic, social, or environmental uses. It concerns both domestic animals (e.g., cattle, sheep, poultry) and species raised in captivity for specific objectives (e.g., aquaculture, beekeeping, or laboratory research). Breeding also involves managing resources and living conditions to ensure animal welfare and productivity.

III.3-Plant cultivation

Plant cultivation refers to the techniques and practices used to grow and maintain plants, whether for food, decoration, scientific research, or other purposes. It includes activities such as soil preparation, sowing, planting, watering, fertilization, pest and disease control, and harvesting. Crops can be grown in open fields, greenhouses, hydroponically (soil-free), or in controlled environments, depending on the objectives and growing conditions.

IV.4-Dissection

Dissection is a scientific method that involves opening and examining the internal structures of an organism, whether plant or animal, to study its anatomy, organization, and functions. This practice is used in education, medicine, and research to directly observe organs, tissues, and their arrangement. It requires specific tools, such as scalpels, scissors, and forceps, and a methodical approach to ensure accurate observations, while respecting ethical considerations regarding the use of organisms.



-The mouse is placed on the dissection tray in a dorsal position, with the ventral side facing the operator.

-The limbs are extended and secured with pins. The preputial opening in males or the urinary opening in females is identified, and a small incision is made in the skin using fine scissors.

-A grooved probe is then gently inserted through the opening toward the head; it serves as a guide for the scissors to prevent damage to the muscles and internal organs. The incision is then continued.

-At the neck level, particular care must be taken with the salivary glands, which are very superficial.

-The same procedure is used to open the skin at the level of the forelimbs and hindlimbs.

-Carefully separate the skin from the underlying muscles by hand, then fold it back to the sides and secure it with pins.

Figure 41 : Steps of mouse dissection

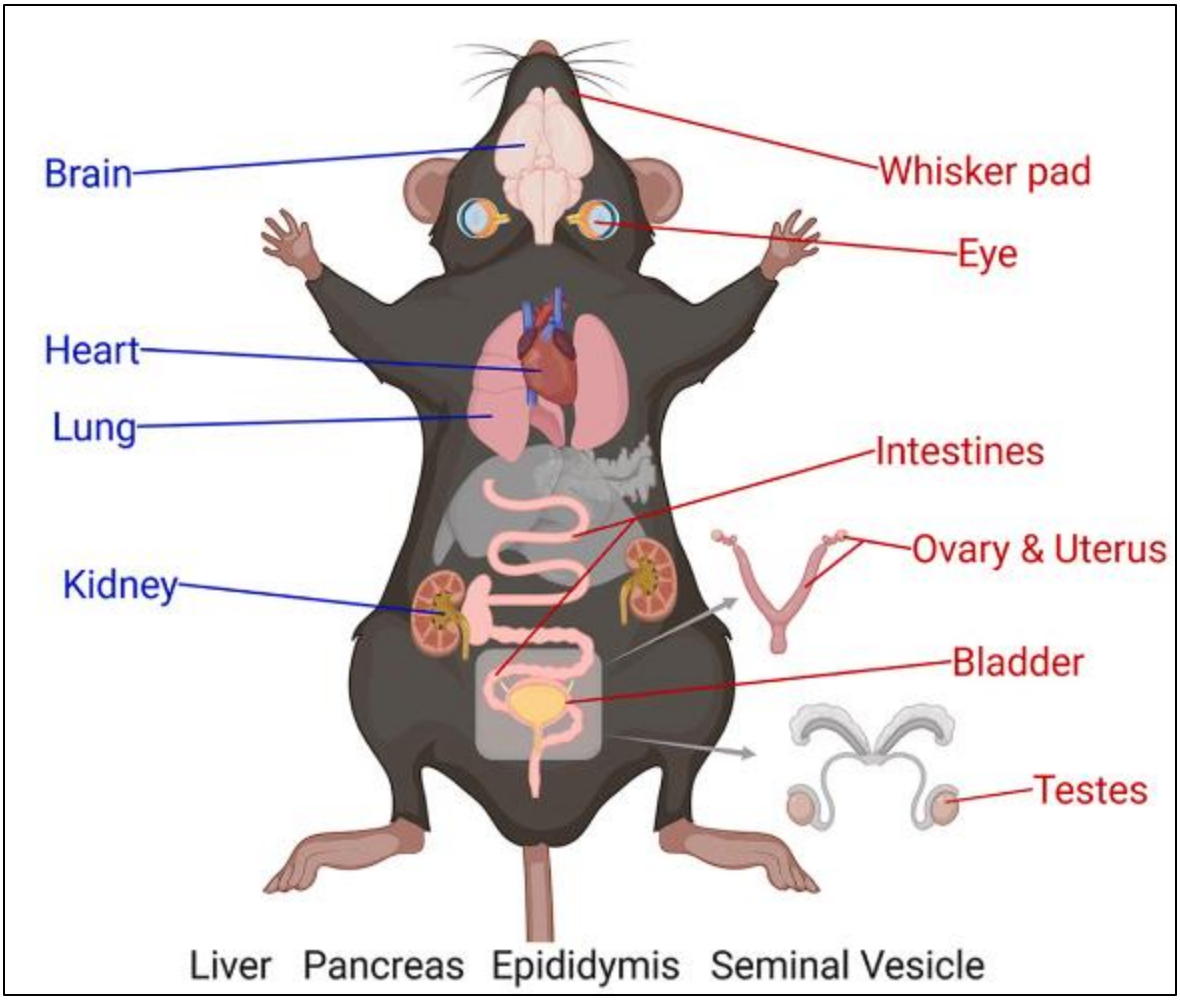


Figure 42 : Mouse anatomy (Panel Jennifer et al., 2025)

Accessing Demographic Parameters of Animal and Plant Populations

V- Accessing demographic parameters of animal and Plant populations

1- Population

A population is a group of individuals of the same species living in the same geographic area (biotope) at a given time and interacting with each other, notably through reproduction. These individuals share common traits and are subject to the same environmental conditions, which influence their dynamics (birth, mortality, immigration, emigration) and evolution.

1.2-Population structure

Population structure refers to how individuals are distributed according to various criteria, influencing the organization and functioning of the population. The main components include:

1. **Age structure:** Distribution of individuals into age classes (young, adult, old), which affects population growth and reproductive potential.
2. **Sex structure:** Proportion of males and females, important for understanding reproductive dynamics.
3. **Density:** Number of individuals per unit area or volume, reflecting the concentration of members in a given space.
4. **Spatial distribution:** How individuals are arranged in their environment, which can be uniform, random, or clustered, depending on available resources and social or environmental interactions.
5. **Genetic diversity:** Variation of genetic traits within the population, influencing its adaptability to environmental changes.

These elements interact and are influenced by ecological, biological, and behavioral factors, playing a key role in population dynamics and survival.

2-Main ecological parameters of populations

2.1 Methods for studying population size

- **Direct counting:** Counting individuals at a given time ("t").
- **Population estimation:** Often involves sampling strategies.
- **Trapping methods:** For sedentary populations.
- **Marking, capture, and recapture methods:** Individuals are captured, uniquely marked (e.g., coloring, rings), and released. After a period, a new capture is performed, and the ratio of marked to unmarked individuals allows estimation of population size.
- **Direct observation:** Counting visual contacts (e.g., large mammals) or auditory contacts (e.g., nesting birds).

3-Descriptive parameters of a population

Population density is expressed as the number of individuals per unit area.

- **Gross density:** Total population size ÷ total area of the studied biotope.
- **Ecological density:** Number of individuals in the habitat actually available ÷ area of the habitat available to the studied population.

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