**Haut du formulaire**

**Scientific methodology and techniques for studying living thingsBas du formulaire**

**Haut du formulaire**

**First part: methods for studying cell morphology**

**I. Cytological methods**

The cells are very small and very complex in organization. The study of their structure, their chemical composition and their functioning (physiology) required the development of appropriate tools and techniques which were perfected as scientific and technological progress was made in various fields. Advances in microscopy have pushed the boundary between the visible and the invisible. Using an electron microscope, we can even obtain images of crystalline gold atoms.

Today, medicine and biology can no longer do without the microscope.

Three approaches are developed to study the various aspects of the cell:

- morphological techniques.

- chemical and biochemical techniques.

- physiological techniques.

**Bas du formulaire**

These techniques are all based on the use of optical and electron microscopes; the manipulations are justified by the two requirements of the microscopic examination:

-The objects to be examined must be thin

- Their different elements must present a certain contrast.

**Haut du formulaire**

**1. Microscopy**

Microscopy is a set of techniques for obtaining an image of biological structures. The principle is in all cases the same: a wave is sent to the preparation or emitted by the preparation. This wave is captured by a lens which focuses it and passes through an eyepiece which creates an observable image. This image is either observed with the naked eye, photographed, or recorded by CCD camera and stored on a computer for reprocessing.

Today microscopy is divided into two large groups, different by the nature of the elementary particle involved: the optical microscope, also called photonic, because it uses photons and the electron microscope which uses electrons to study the object. In order to study structures we use a certain number of techniques: preparation of thin sections, negative staining, metallic shading , ….. etc.

**Haut du formulaire**

**1.1. Light microscopes**

This technique is the oldest used. It is also the one of which there are the most variations. The principle is as follows, the preparation is illuminated by a lamp. The molecules to be observed will interact with light in several ways:

- by absorbing certain wavelengths of light. This is direct light microscopy.

- either by causing a phase shift of the different light rays. This is phase contrast microscopy.

- or by emitting light at a wavelength other than the original one. This is fluorescence microscopy

The optical microscope is an optical instrument equipped with an objective and an eyepiece which makes it possible to magnify the image of a small object (which characterizes its magnification) and to separate the details of this image (and its resolving power) so that it is observable by the human eye. It is used in biology, to observe cells, tissues, in petrography to recognize rocks, in metallurgy and in metallography to examine the structure of a metal or an alloy.

Haut du formulaire

It should not be confused with the binocular magnifying glass which does not require thin flat samples.

This microscope has the advantage of giving a general view of cells or tissues and also of allowing the examination of living cells; but the resolving power of the optical microscope cannot exceed 0.2 μm, the magnification being a maximum of 1000.Bas du formulaire

**a: Presentation of the optical microscope**

An optical microscope is generally composed of a stand (foot) which ensures the stability of the device, an optical tube along which there is a system of glass lenses and comprising at its ends an eyepiece for collecting the image and objectives used to magnify the image of the preparation a certain number of times, a stage (object holder) pierced with a hole and equipped with clamps to immobilize the slide and a light source illuminating the preparation. (See practical work on microscopy).

The microscope is characterized by:

- **​​its magnification or power**: Equal to the product of the magnification (or power) of the objective and the eyepiece. The greater the magnification of the objective, the closer the objective must be to the object to be observed.

- **its resolving power**: The resolution of a microscope refers to its ability to separate very similar details. Regardless of the sensor used and the aberrations or imperfections of the lenses, the resolution of the optical microscope is fundamentally limited by the diffraction of light.

**Resolution or resolving power (RP) is defined as the minimum distance separating two distinguishable points. In humans, the RP is 0.1 mm at a distance of 25 cm. The human eye can only distinguish two objects at 25 cm that are only 0.1 mm apart. Beyond this distance, the image of the two objects will be unique or null. The RP of the optical microscope is 0.2 μm.**

**b: Principle of operation of the microscope:**

Two types of observations can be carried out in microscopy: observation by transmission for the optical microscope and for the transmission electron microscope and observation by reflection for the scanning electron microscope.

So the microscope works in:

**Transmission:** the sample is crossed by photons and electrons; the glass lenses (MO) or the electromagnetic fields (MET) allow an image to be obtained which is taken up by the eyepiece (MO) or fluorescent screen (MET).

**Reflection:** the microscope only captures the rays reflected by the walls of the preparation. This type of microscopy gives an image of the surface of objects and not of their internal structure. Since the intensity depends on the orientation of the walls in relation to the optical system, this gives a "relief" image of the object. They are therefore not applicable to objects without relief such as tissue sections! They require "lateral illumination" of the object. This mode of microscopy is rarely used; it corresponds to binocular magnifiers or stereomicroscopes, the dark-field microscope in optical microscopy and the scanning electron microscope (SEM) in electron microscopy.

**c: Observation conditions in microscopy:**

To carry out an observation in microscopy, two requirements are necessary: ​​the thickness of the sample and the contrast.

**The thickness of the sample:** for observation by transmission, the sample must be thin to allow the passage of the incident beam of photons or electrons, hence the need to make very very thin sections. The sections required in (MO) vary between 2 μm to 10 μm and from 0.03 μm to 0.05 μm

**Contrast:** Observation by transmission is only possible if certain regions of the section absorb photons or electrons more than others (contrast effect). As a general rule, cellular constituents have low natural contrasts, hence the use of certain devices such as optical assemblies that amplify natural contrasts, such as the phase contrast microscope or selective vital dyes (MO) or even heavy metal salts such as lead salts (ME).

d: Types of optical microscopes: A number of microscopes, each with special optical assemblies, have been developed to enable the observation of cells under certain conditions and to improve their quality. The development of these microscopes mainly meets 2 objectives

- increase contrasts to better visualize subcellular structures

- improve resolving power (see smaller and smaller details!)

Among these microscopes, we have:

**a) Dark field microscope:**

It allows certain details to be revealed when observing living cells, by increasing natural contrasts. In this type of microscope, the light source is oblique to the cell preparation. A special condenser illuminates the preparation at a grazing incidence, only the reflected rays are captured by the objective: the background of the observation field is black, and the smallest object appears brightly lit. This allows the observation of objects whose size is at the extreme limit of the resolving power, and which would go unnoticed with ordinary techniques. But since these objects become light sources, their shape and size cannot be properly appreciated.

**b) Phase contrast microscope:**

This type of microscope is widely used for observing living cells. Its principle is based on the amplification of natural contrasts by taking advantage of the differences in refractive indices between organelles; differences that it transforms into differences in light intensities that are then visible to the eye. The basis of this transformation is the interactions between light waves: we speak of interference. This microscope is a good tool for observing the movements of cells and their organelles such as mitochondria, chromosomes, and for following the stages of processes such as mitosis for example. The images of the observation can be recorded by video camera, the films are then projected on a television screen.

**c) UV (Fluorescence) Microscope:**

Generally speaking, fluorescent molecules absorb radiation at a given wavelength and emit radiation at a higher wavelength. Examples of this type of substance are substances called Fluorescein, which fluoresces green, and Rhodamine, which fluoresces red. These are widely used substances in cell biology. This microscope is similar to a regular light microscope, except that it is equipped with a UV source (UV lamp) and a filter system that allows the appropriate UV wavelength to be selected for each substance. It is most often used to detect specific proteins or other molecules made fluorescent by coupling to a fluochrome; for example, the presence of insulin in a cell can also be detected with fluorescein-labeled anti-insulin antibodies.

**d) Polarized light microscope:** It can detect birefringent structures that have a particular molecular organization, such as microtubules and chloroplasts and plant cell walls. Light photons passing through certain materials such as Polaroid filters or certain crystals (Nicol) emerge "polarized": they only vibrate in a single plane. If a second Polaroid filter or a second Nicol is placed in their path, it is possible to completely stop them by rotating this second filter and obtain total extinction of the beam. ("Crossed" Nicol) If an object active on polarized light is placed between the two crossed filters, such as an organic substance with asymmetric carbon or an ordered molecular arrangement, the polarization plane is deflected and the extinction is lifted, the light from the object passes through the second filter. It will be necessary to rotate the second filter to obtain extinction again. A polarized light microscope is equipped with a first filter at the condenser and a second filter in a crossed position in the optical tube.

**e) Scanning microscope:** In this type of microscope, the object is illuminated by a finely focused laser beam that scans rapidly at a single level illuminating only a thin plane of the object, we speak of optical sections. The preparations are often treated with fluorescent dyes and the light emitted by the illuminated optical section gives an image on a video screen. Photographic sections of a human brain taken by a scanner are an example of this type.

**B: ELECTRON MICROSCOPY**

The electron microscope is much more recent: the first was built in 1931 by Max Knoll and Ernst Ruska, the latter of whom received the Nobel Prize in Physics in 1986 for this invention. Then it became widespread from the 1960s. The resolution of an electron microscope can reach 2 angstroms, but generally the best microscopes reach only 20 angstroms. The operating principle of an electron microscope is somewhat similar to that of an optical microscope except that instead of photons this microscope works with electrons, the beam is produced and accelerated by an electron gun (cathode and pierced anode). The entire device is placed in a vacuum. The glass lenses are replaced by electromagnetic coils (electromagnetic "lenses") which are the only ones capable of focusing the electrons and creating images.

With these microscopes, only dead cells can be examined, but the resolving power is of the order of a few A°. This gives us access to the ultrastructure of organelles.

**1: Types of electron microscopes**

There are two variants of electron microscopy:

- transmission microscopy

- scanning microscopy

**a) Transmission electron microscope:**

This is the most efficient technique. In principle, it is similar to direct light optical microscopy. The electron beam is emitted by an electron gun, focused on the preparation using electromagnetic lenses and passes through it, they are more or less absorbed (the preparation is said to be more or less electron dense), the image is formed behind the preparation on a fluorescent screen similar to those used in black and white televisions. Apart from the fact that the electron absorbers are heavy metals, the same development techniques as for direct light microscopy can be used.

**b) Scanning electron microscope:**

Although the resolution is lower than the previous one, this technique gives absolutely spectacular images, in pseudo 3D. The flow of electrons scans the surface of the object previously covered with a metallic layer. It is the secondary electrons, returned by the metallic surface, which are used to provide an image. This device allows for greater depth of field, but its resolving power is lower than that of the transmission microscope. It provides information on the three-dimensional appearance of cell surfaces, for example.

**II. METHODS FOR STUDYING THE BIOCHEMICAL COMPOSITION OF CELLS**

**1. Cellular materials**

**1.1. Whole cells or cell sections:** The microscope allows us to observe the cells of a tissue, but under very strict conditions: the object to be examined must be transparent to light. Biological objects are rarely transparent, with the exception of naturally very thin objects: suspensions (blood, etc.) or cell cultures. They must therefore be cut into very thin slices (sections) of the order of 5 to 10 microns. To do this, the object must be "hardened" using various physical or chemical techniques. But cutting would cause irreparable damage to tissues and cells and the observation would not correspond to the reality of living cells: physical separation of close structures more or less driven by the cutting tool, release of lytic enzymes (mainly proteases) contained in specific compartments (lysosomes, peroxisomes, etc.) it is therefore necessary to first "immobilize" the structures in a state as close as possible to the living state and inactivate these enzymes: this is the aim of a whole series of manipulations which will bring the biological objects to the appropriate examination under the microscope.

**A. SECTIONING TECHNIQUES**

For a morphological examination of cells or biological samples by microscopy, sample preparation procedures are necessary.

**􀂙 Examination of samples by optical microscopy**

**􀂾 Examination of living cells:**

They can be examined without preparation, but in a very limited number of cases. This can only be isolated cells, naturally or in culture.

An animal cell without an exoskeleton dehydrates very quickly in air. Its observation can only be carried out in a liquid medium. Observation in a culture medium allows the cellular physiology to be maintained. But culture dishes prohibit useful magnification objectives (maximum X5). The trick is to reverse the assembly of the optical system: the dish is illuminated from above (current condensers allow the light to be focused on the bottom of the culture dish) and the observation is made through the bottom: inverted microscope.

**How can we properly observe living cells?**

We must use techniques that increase contrast without causing toxicity to the cell.

**a) chemical methods, vital dyes:**

Almost all dyes are very toxic to cells, a few rare dyes do not have this disadvantage. They increase the absorption contrast of certain wavelengths, giving a color to the structures that retain them. We can cite: **Janus Green B** specific to mitochondria, **Trypan blue**, which cannot penetrate living cells, but which colors dead cells (trypan blue exclusion test): it is widely used to evaluate the vitality of cells.

Exclusion dyes derive from a technique used by microbiologists who visualized yeasts by contrast by dispersing them in India ink

**b) physical methods, phase contrast microscope:**

this microscope increases the contrast of objects. It is the only way to observe cellular movements and film them.

**Examination of dead cells:** It is necessary to carry out essential manipulations to obtain thin and contrasted objects. In fact, we can only examine colored ones; it is also necessary to obtain thin sections of these cells and therefore to include them beforehand in relatively hard substances, they must first be fixed, to avoid any alteration likely to occur during manipulations. The sequence of these manipulations is therefore as follows: Sampling, fixation, inclusion, sectioning and staining.

**a) Sampling:**

In the medical field, samples are taken in clinics, hospitals, private practices or specialist doctors. They are performed by surgeons.

• There are four major categories of sampling:

o Smears: scraping (from the cervix, etc.),

o Biopsies: fragments of tissue or organ,

o Whole organs,

o Various effusion fluids (pleural, ascitic, pericardial, etc.).

There are also more sophisticated sampling techniques: by excision, puncture or microdissection.

**b) Fixation:**

This is the action of killing cells, avoiding any agonic phenomenon, so as to preserve the structures in a morphological state as close as possible to the living state. Good fixation must avoid any artifact: appearance of a new structure (by coagulation), disappearance of a structure normally present (by solubilization), deformation or displacement of cellular constituents (by crystallization). It can be fixed using chemical processes: Alcohol, formalin, acetic acid, etc. or by physical processes, such as sudden freezing (best fixative).

**c) Dehydration:**

To dehydrate tissues, they are immersed in alcohols of increasing degrees, 70°, 80°, 90°, 100°, for the time necessary for the concentrations to equilibrate. Paraffin is not miscible with water, the anatomical piece must be completely dehydrated before inclusion in the paraffin. Paraffin is also not soluble in the alcohol used for dehydration. Therefore, a double substitution is carried out.

􀂃 Water is replaced by alcohol (Dehydration)

􀂃 Alcohol is replaced by toluene (Substitution)

**d) Inclusion:**

The section can only be made in a fairly hard substance; this is why the tissues are impregnated with an embedding substance, generally paraffin; since alcohol is not perfectly miscible with paraffin, the dehydrated tissue is immersed in an intermediate organic solvent miscible with alcohol and paraffin, xylene, then in paraffin kept liquid in an oven between 50 and 60°C. It is then cooled and a block of hardened paraffin containing the tissue to be examined is obtained. This impregnation requires dehydration and a substitution of water with alcohol, the solvent for paraffin. In fact, the inclusion will only be satisfactory if the piece to be cut contains neither water nor an intermediate solvent (alcohol).

**e) Section (microtomization):**

The paraffin block is cut into thin slices using microtomes, which are devices for cutting paraffin blocks into sections from a few microns to a few tens of microns. The friction forces between the knife and the block heat the paraffin and supercool it, which allows the sections to be glued together: a ribbon of serial sections. They are collected on glass slides (object holders) previously coated with an ovalbumin solution which sticks them to the slide when drying. Currently, chemically treated glass is used.

**f) Rehydration:**

The sections glued to a glass slide are deparaffinized using an organic solvent and brought back to water by baths of alcohols of decreasing concentrations. This allows them to be stained, since the majority of dyes are soluble in water or alcohol.

**g) Coloring:**

Biological objects cut and examined by transparency are not or only slightly colored: they offer very little contrast, therefore visibility, and no detail can be perceived. It is necessary to reinforce the color contrast of the different organelles or to color them better. This glass slide is then immersed in a dye.

There are many natural dyes available, which fix on this or that structure of the cell, for example, methyl green colors chromatin green.

Two categories of dyes are used: The most common are: hematoxylin, which colors cell nuclei in purplish blue, Eosin, which colors cytoplasms in pink Blues (methylene blue, toluidine blue) are also used routinely.

**- Examination of samples in electron microscopy**

The manipulation sequence is similar to that outlined for optical microscopy.

**a) Fixation:**

even more demanding (artifacts are more visible), it is carried out with special fixatives, such as: osmium tetraoxide OsO4 and glutaraldehyde C5H8O2. These are the two main chemical fixatives used in electron microscopy.

**b) Dehydration:**

it follows the same principle as in optical microscopy; but it is delicate because the tissues must be preserved down to the molecular level.

**c) Inclusion:**

it is carried out in a very hard environment, in plastic materials such as resin. Once cooled, hardened by polymerization, a solid sample is obtained.

**d) Section:**

carried out on an ultramicrotome, it provides even thinner slices, 50 nm.

**e) Coloring:**

it is rather an impregnation (there is only black and white in electron microscopy) by heavy metal salts, such as lead or uranyl salts, which increases the contrast of cellular structures.

**B. REPLICA TECHNIQUE**

It is generally applicable to SEM and is carried out in 3 stages: freezing the sample, cryofracture and obtaining replicas.

**Examination of replicas after freeze-fracture and freeze-etching:**

This technique allows a topographic impression (replica) to be made after fracturing a frozen sample. It allows the production of a single or double replica (molding of both sides of the fracture). The tissue is fractured after fixation at low temperatures; the fracture line presents reliefs due to the heterogeneity of the cellular constituents. These are accentuated by the projection at a grazing incidence (shadowing) of a thin metal layer. This is then detached and constitutes a replica that can be examined under a microscope. We then have an idea of ​​the reliefs of the surface of an organelle or even of a membrane if the fracture has delaminated it (tangential section).

It is generally applicable to SEM and is carried out as follows:

Freezing the sample, freeze-fracturing, stripping, shading and obtaining the replica. The collected tissues are frozen quickly without fixation or after fixation. In order to avoid damage caused by the formation of ice crystals, the tissues are impregnated with substances such as glycerol, then frozen quickly in very low temperature liquids such as liquid freon, whose melting point is -150°C or by placing the sample in contact with a block of metal cooled in liquid helium. When the tissue is frozen, it is often observed by the freeze-fracture replica technique, illustrated in the figure below. Small fragments of tissue are placed on a small metal disc and frozen quickly, the disc is then placed in a special holder and the frozen tissue block is struck by a blade, causing from the point of contact a crack that cleaves the tissue into two parts.

When the fracture plane passes through a cell composed of very diverse organelles of different composition. These structures tend to deflect the fracture plane, upwards or downwards, causing protrusions, depressions and ridges on the surfaces that reflect the contours of the protoplasm crossed. In other words, the surfaces exposed by the fracture provide information about the contents of the cell. The goal is to make this information visible. To achieve this, the replica technique uses the fracture surface as a mold on which a layer of heavy metal is deposited. The metal is deposited on the surface of the frozen tissue that has just been exposed in the same enclosure that was used to produce the fracture. The metal is deposited at an angle that accentuates the local topography by shadowing. A layer of carbon is then deposited on top of the metal layer directly from the top, rather than laterally, so as to obtain a uniform layer of carbon that cements the metal areas into a continuous film. When a cast of the surface has been obtained, the tissue that served as a model can be melted, removed and discarded; it is the replica of metal and carbon that is placed on the grid and observed in the electron beam.

Therefore, this technique is particularly suitable for studying the interior of membranes. Freeze-fracture replication is, in itself, an extremely useful technique, but it can be used to provide even more information when a freeze-etching step is added. During this step, the frozen and fractured object, still in the cold chamber, is placed under vacuum at a high temperature for one or a few minutes: a superficial layer of ice evaporates (sublimation). After the water is removed, the surface of the structure can be covered with a heavy metal.

