Cell Biology Course

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Chapter 02
Methods of studying the cell

Introduction

The cell is the fundamental structural and functional unit of living organisms and because of its small size, cell biology first needs to obtain a good quality enlarged image of it; this has required the development of tools such as microscopes and appropriate techniques that have been improved over time.

Microscopy:

Microscopy is divided into two large groups, differing 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 observe even finer details, it is necessary to increase the resolution, which is generally proportional to the wave length of the radiation used to interfere with the structures studied.There are two main types of microscopes according to their resolution:Optical microscopes and electron microscopes.

Optical microscopy

The optical microscope is an optical instrument equipped with an objective and an eyepiece that allows the image of a small object to be magnified and the details of this image to be separated so that it can be observed by the human eye.All microscopes are characterized by their resolving power (resolving power, i.e. the appreciable limit distance between 2 points and also the resolution limit).A transmission photonic microscope distinguishes objects at most 0.2 µm apart.

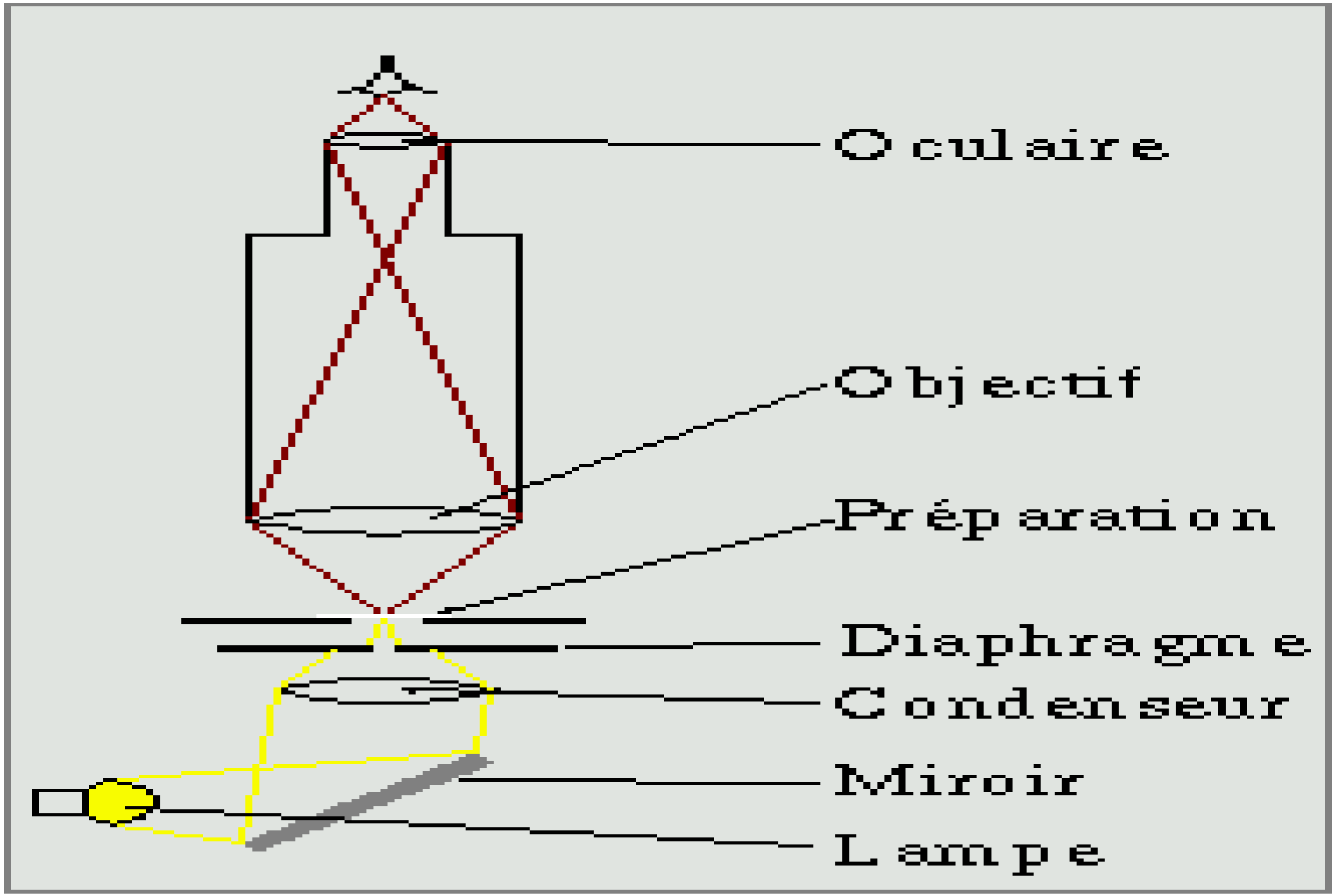
Definition of light microscope:

- It is a magnifying device that relies on light and lenses to magnify the material. It is characterized by being low-cost, more widespread in the fields of education and medicine, and is characterized by providing the ability to monitor the activities of living cells.

The magnification of an object is the product of the magnification of the objective by that of the eyepiece
Optical microscopes (light or photonic) allow the observation of living or dead cells, thanks to very fine sections of fixed preparations, it thus gives a general view of the cells or tissuesOptical microscopes use visible light and the resolving power of the photonic microscope reaches its theoretical limit at 0.2 µm, the magnification being at most x1000.

Figure 1. Descriptive diagram of an optical microscope

Figure 1. Schéma descriptif d'un microscope optique



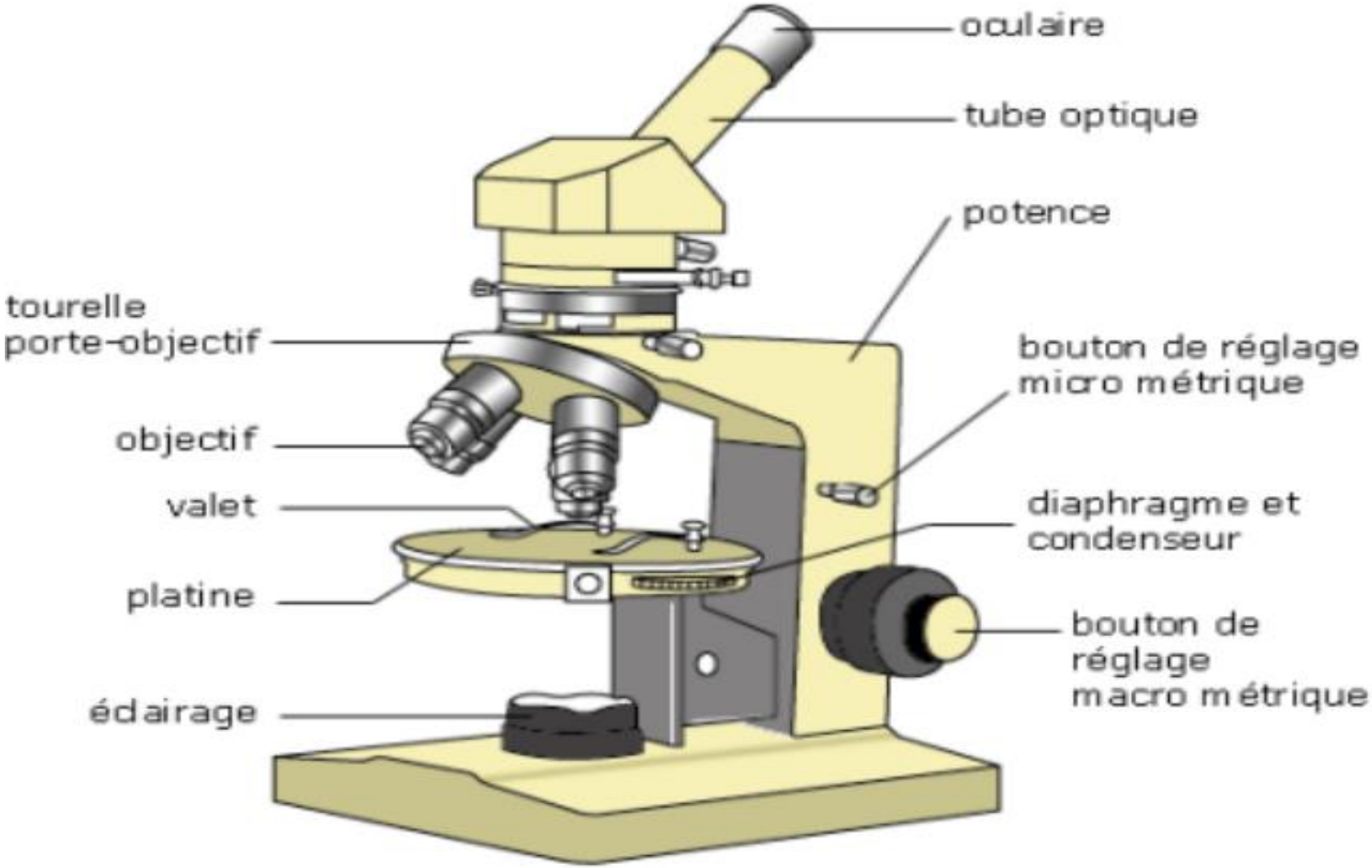


Figure 1 : Schéma d'un microscope optique monoculaire

Figure 1: Schematic of a monocular optical microscope

- **Types of light microscopes:**
- **Simple light microscope:** It magnifies the object with a simple magnification power; because it consists of a single lens with two sides.
- **Dissecting microscope:** This device consists of two eyepiece lenses and objective lenses, and its magnification power for objects ranges from 6 to 50 microns.
- **Compound light microscope:** This device consists of a magnifying lens in the eyepiece lens, and another in the objective lens, and its magnification power for the object ranges from 40 to 1000 times, and it is considered one of the most important devices in biology.
- **Inverted light microscope:** It differs in its design from other light microscopes in terms of the objective lens placed from the bottom to magnify the sample, and the light is controlled as needed.
- **Digital microscope:** This type is characterized by the presence of an added camera, and allows the display of the magnified image on the computer screen, data show, and television, and its magnification power reaches 400 zoom.

- **Phase contrast microscope:** This microscope includes a disk with a circular cavity in the form of a ring, called the phase ring, as well as the phase plate, and is used to study unstained living cells. This type of microscope is widely used for observing unfixed living cells.Its principle is based on the amplification of natural contrasts by taking advantage of thedifferences in refractive indices between organelles; which it transforms into differencesin light intensities that are then visible to the eye.
- **Fluorescent or fluorescent microscope:** It is called this because it depends on magnifying objects on a fluorescent dye that absorbs light and works to radiate it at a greater wavelength.
- **Dark field microscope:** It is characterized by the opaque slide floor, and the light is in the form of a ring on the living organism whose activity is to be monitored, and it is special for examining a type of bacteria.

Polarized light microscope

It depends on the polarization of the light beam to distinguish between materials with double refractive power, and the most important fields in which it is used are geology, medicine, and biology.

It allows to detect birefringent structures that have a particular molecular organization, such as microtubules and chloroplasts and plant cell walls.

An object active on polarized light, such as an organic substance with an asymmetric carbon or an ordered molecular arrangement, is placed between two crossed filters, the polarization plane is deflected and the extinction is lifted, the light coming from the object passes through the second filter.

It will be necessary to rotate the second filter to obtain extinction again.

The UV microscope (= fluorescence)

This type does not have eyepieces, but rather relies on short-wave ultraviolet rays, as well as cameras that photograph the object and then magnify it later.

This microscope is similar to the ordinary photonic 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 an Anti-insulin antibody labeled with fluorescein.

is generally used for unstained samples. The image obtained has a dark, almost black, background (the observation background)

2. Electron microscopy

- Electron microscopes use electron beams that are charged, have mass, and behave like a wave. The more the electrons are accelerated, the shorter the wavelengths and the higher the resolution.These microscopes are equipped with electromagnetic lenses. The resolving power of the electron microscope is 0.2 nm and is 1,000 times higher than that of the optical microscope, the magnification being at most x 100,000.

The resolution of an electron microscope can reach 2 angstroms. The operating principle of an electron microscope is a bit like 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 glass lenses are replaced by electromagnetic coils (electromagnetic "lenses") which are the only ones capable of focusing electrons and creating images. With these microscopes we can only examine killed cells, but the resolving power is of the order of a few A°.

We will therefore have access to the ultra structure of organelles.

Types of electron microscopes

There are two variants of electron microscopy:

Transmission microscopy Scanning microscopy

Transmission electron microscope:

As the electron beam passes through the sample and its atoms, it produces different types of radiation. In general, only the transmitted electrons are then analyzed by the detector, which translates the signal into a contrasted image.

Scanning electron microscope:

Although its 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 has a resolving power that is lower than that of the transmission microscope.

Figure 2. Comparative diagrams of the paths of light rays and electrons in a photon microscope and in an electron microscope.

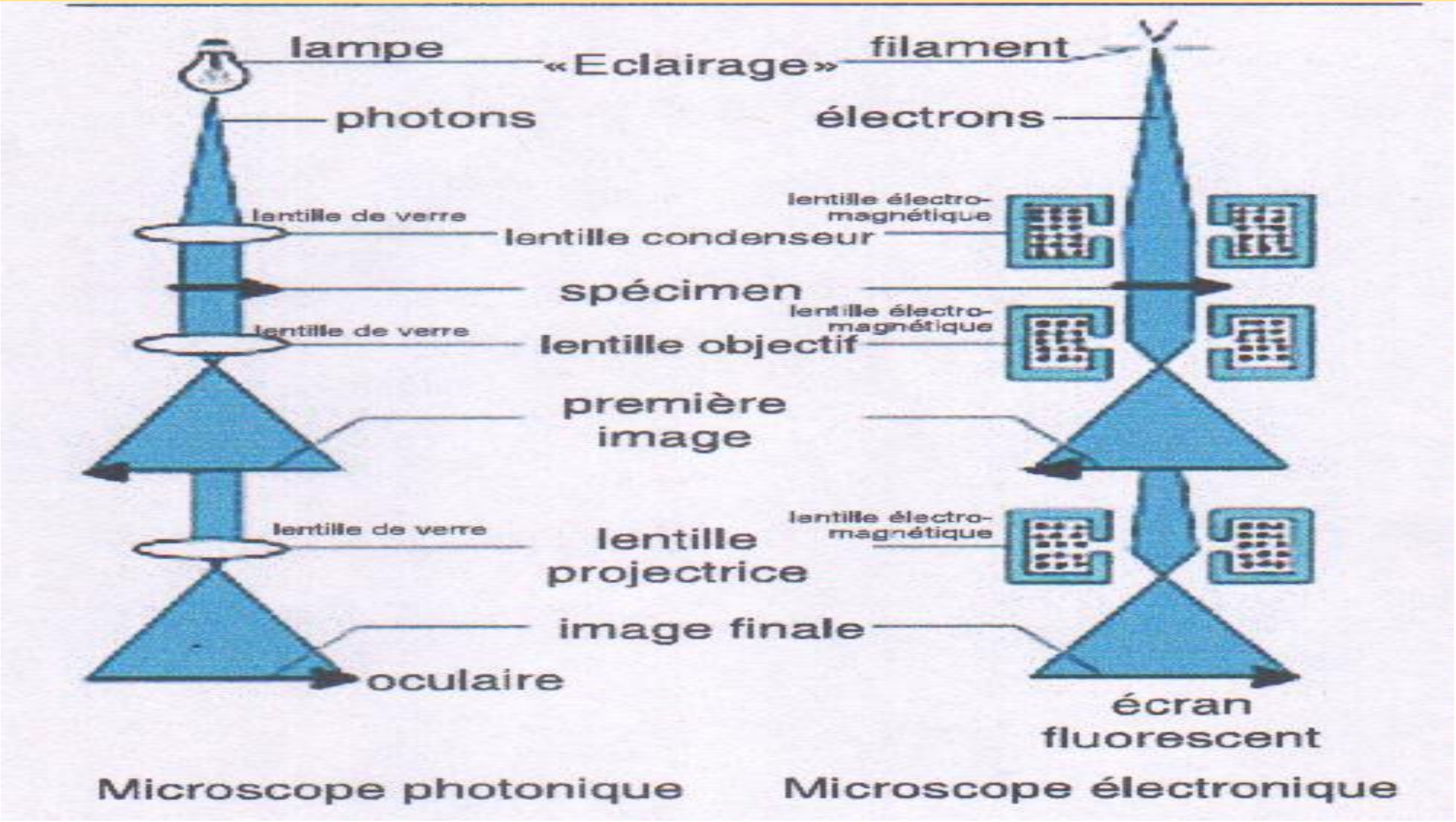


Table 2: The main differences between MO and ME

	MO	ME
Source d'énergie Rayonnement	Lampe électrique photons	Filament de tungstène porté à incandescence Electrons libres accélérés dans le vide pour traverser ensuite l'échantillon
Système optique	Lentille de verre	Lentille électrique Lentille magnétique
Pouvoir de résolution= pouvoir séparateur	0.2µm	0.2nm (2Ä)
Epaisseur de l'échantillon	2 à 10µm	300-800Ä
Grossissement	40 à 2000	500.000(MET)

a) Examining samples using light microscopy

❖Examining living cells:

Techniques that increase contrast without causing cell toxicity must be used.Chemical methods, vital dyes:Almost all dyes are highly toxic to cells; a few rare dyes do not have this drawback.These include:

❖Janus Green B, specific to mitochondria

❖Trypan blue, which cannot penetrate living cells but stains dead cells (trypan blue exclusion test): it is widely used to assess cell vitality.Physical methods, phase contrast microscope: This microscope increases the contrast of objects. It is the only way to observe and film cell movements.

Microscopic observation of the cell

- The cell is observed on two levels: outside and inside.

INTERNAL OBSERVATION OF THE CELLA
- Section preparation techniques:

Due to the low penetrating power of electrons, the objects observed must be extremely thin (sections of 50 to 80 nm), which requires specific sample cutting techniques. When the biological material is massive (animal or plant tissues: liver, brain, muscle, etc., or stems, leaves, roots, etc.), it must first be cut into thin, regular slices, which will then be stained. From a 20 µm diameter cell, 200 to 400 ultrathin sections can be cut. The steps of this protocol differ depending on whether the observation is performed under light or electron microscopy.

Light microscopy	Electron microscopy

Fixation:

aims to kill cells while modifying their internal structures as little as possible. For this purpose, various mixtures are used, containing substances known to denature and coagulate mainly proteins: acids, alcohols, aldehydes, certain salts, etc. These fixatives, called coagulants, must be adapted to the nature of the biological material analyzed and the type of staining used subsequently.

Heat, alcohol, formalin, etc

Glutaraldehyde, osmotic acid, etc.

Dehydration: aims to eliminate water from the sample and replace it with a solvent from the medium used for inclusion; it consists of a series of baths in increasingly concentrated alcohols. This operation must be gradual so that the substitution does not cause tissue deformation. A final bath is carried out in a mixture of alcohol/organic solvent from the inclusion medium, to finally have the sample in this pure solvent: xylene, toluene, etc.

Inclusion or Embedding:

Aims to completely impregnate the cells with a hardening substance, which will allow a fine and regular section. This substance, whose molecules actually replace the initial water molecules, is often paraffin or epoxy resin which is liquid at 60 °C and hard at room temperature; soluble in the solvents mentioned above, it penetrates very easily into the tissues. After several baths at 60 °C and hardening of the resin, a "block" is obtained that can be cut correctly and also serves as a means of storing samples.

Paraffin
Synthetic resin Epoxy

Cutting:

aims to produce thin and transparent sections of the included object . A microtome is used for optical microscopy, equipped with a metal razor, which gives serial sections; these are stuck with a gelatin solution on glass slides, dried and deparaffinized with xylene or toluene. Only the organic matter of the section remains on the slide. -Microtome with metal blade of the type -Ultramicrotome with glass or Razor blade

-of the order of 5 to 7 µm diamond -
of the order of 0.1 µm and less

Rehydration:

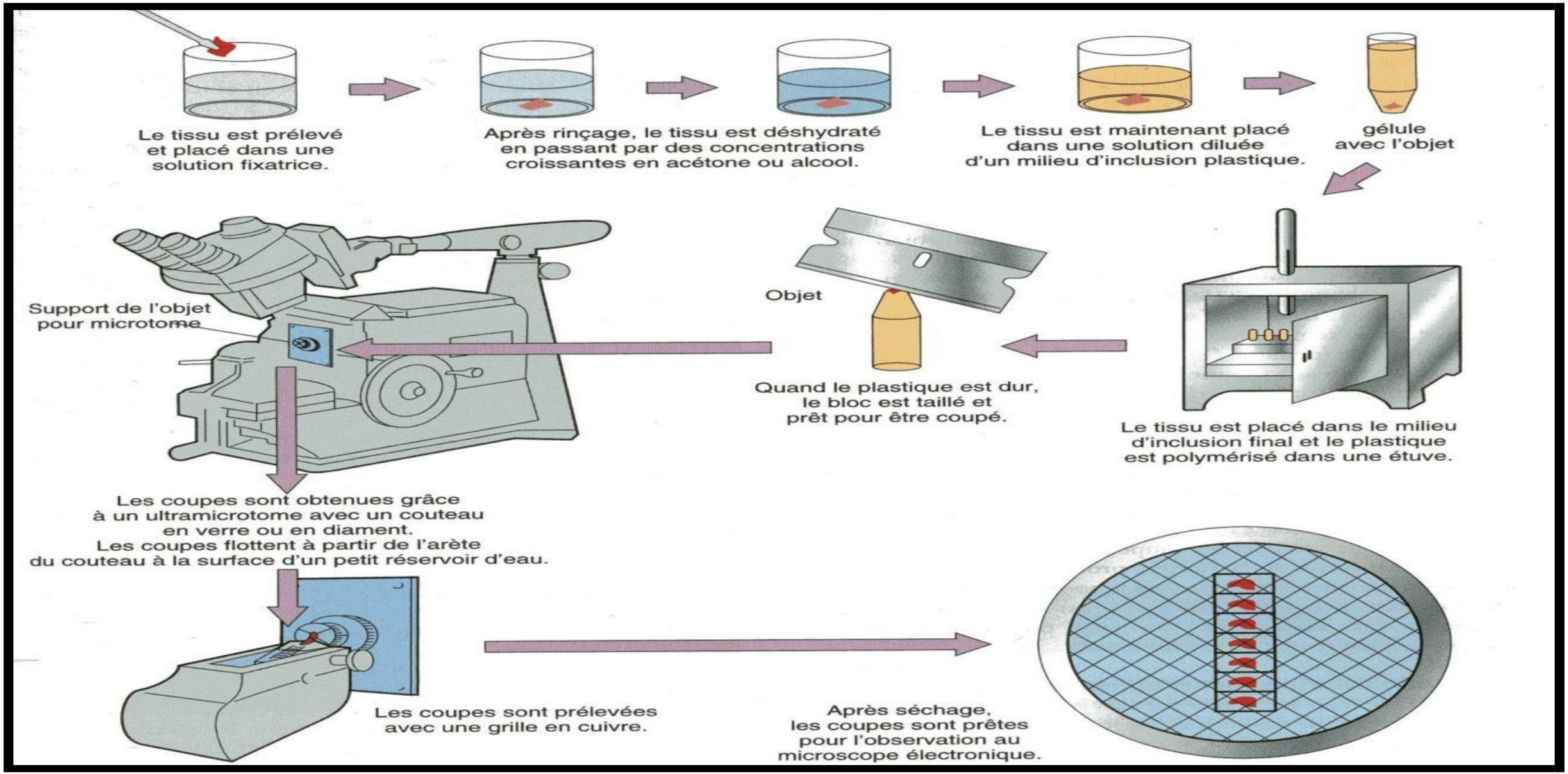
This requires the use of a series of alcohols of decreasing strengths, to arrive at water.

Staining:

Allows to stain the various areas of the biological sample differently. - for optical microscopy, all the dyes used are water-soluble, they color the sample and allow light to pass through.

- for electron microscopy, heavy metals are used to increase the low contrast resulting from the little interaction between electrons and biological surfaces.
- Methylene blue, neutral red... Heavy metal salts: Lead citrate...

Figure. Summary of the section preparation method



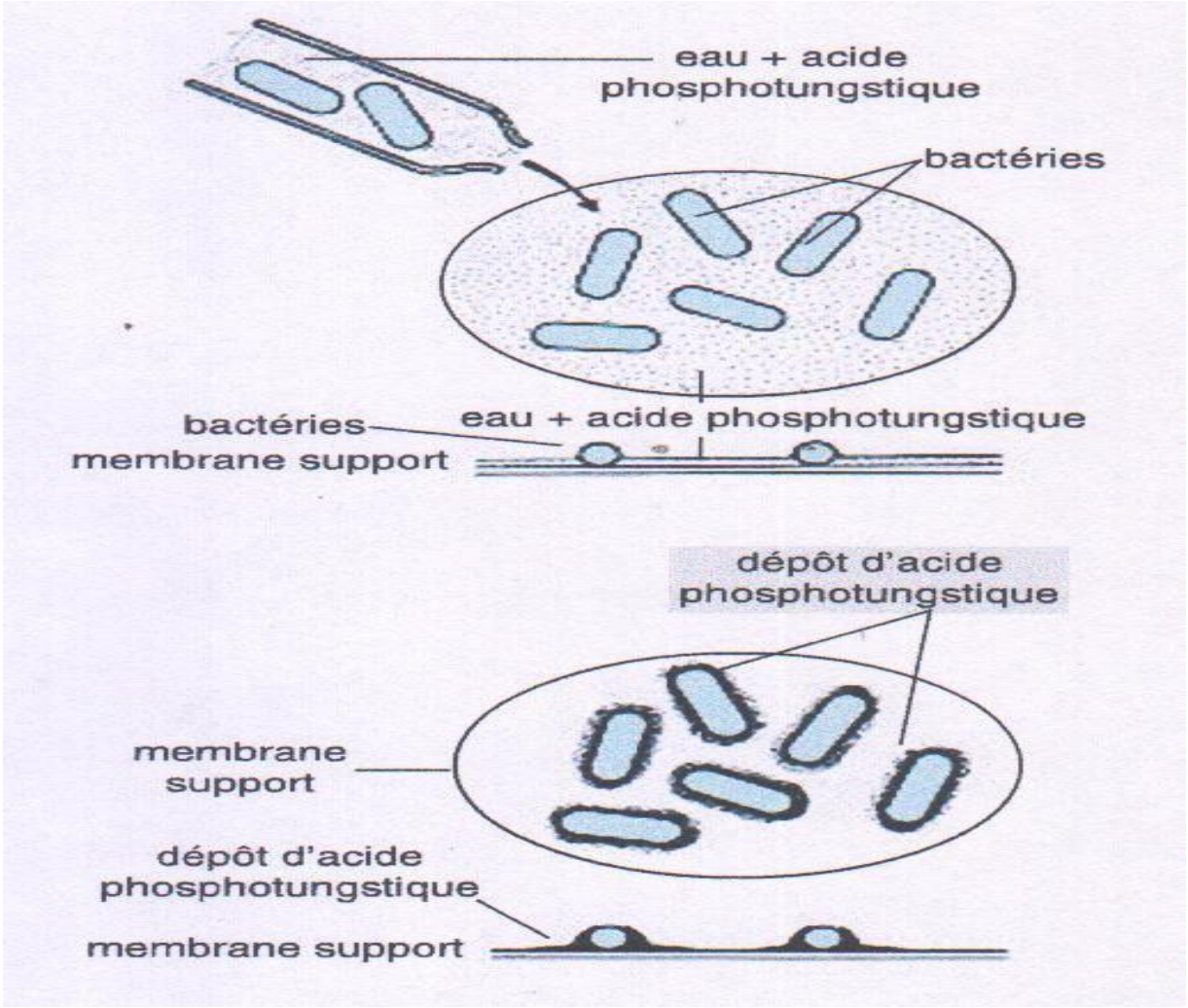
B-Techniques for observing cell shapes and surfaces

There are samples and objects of very small dimensions, of relatively simple shape (molecules, Viruses or isolated cellular elements: organelles or fragments of organelles) with very fine details that do not require cutting, particularly in the case of fibrous structures (protein protofilaments, flagella, tail of certain Viruses, etc.). The observation of the latter under a transmission electron microscope poses a problem of lack of contrast, which we can overcome by using one of these two techniques:

B-Techniques d'observation des formes et surfaces cellulaires

1- NEGATIVE STAINING Principle: consists of placing the object to be studied on a support, then immersing the whole in a solution of heavy metal salt, which is drained. and the traces of solution that remain are concentrated on the corners or edges of the objects.

Figure 5. Principle of negative staining

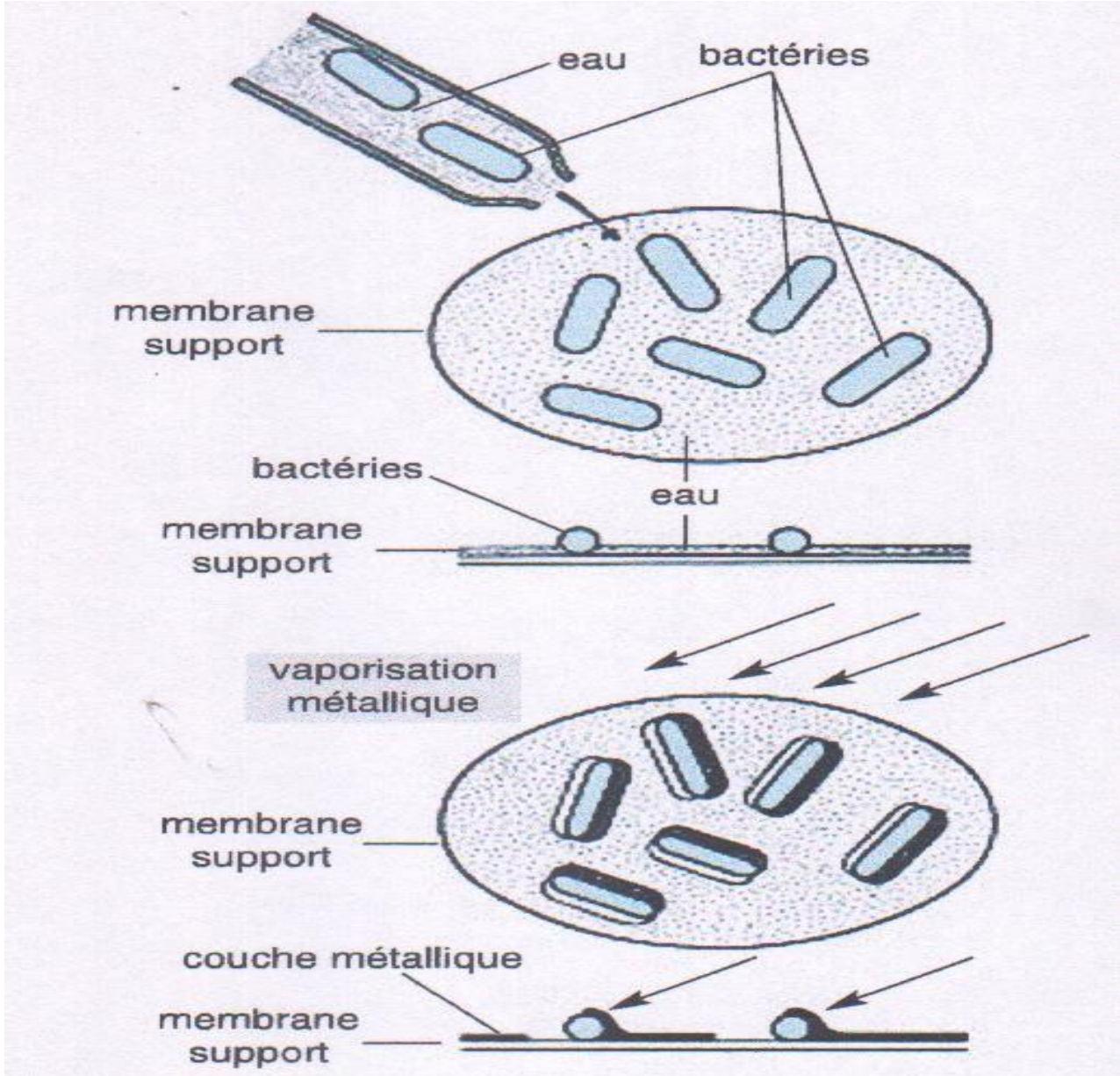


2- METAL SHADING

Principle:

consists of placing the object to be studied on a support, then exposing the whole to metal vapors (obtained by vaporizing a metal electrode under vacuum) and this under incidence to have a shadow formed.When observing massive tissues, and their internal components that are not the result of a cut, but that of a fracture, we speak of cryofracture and cryodescaping.

Figure 6.Principle of metallic shading



3- Cryofracture and cryodescaling

This technique is a development of the so-called metallic shadowing technique developed ten years earlier. It is first based on the very rapid freezing of a very small biological sample (less than 1 mm3), in liquid nitrogen (– 196 °C).The crucial step of the protocol is the fracture, and not the cutting, of the frozen sample, and this at a very low temperature. This cryofracture releases an irregular surface across the sample, and it is this surface that will be observed. The surface of the sample is then stripped by sublimating, under vacuum and at low temperature, a thin film of superficial ice; which has the effect of very slightly increasing the reliefs of the structures (cryodescaling). This is followed by metallic shadowing, under vacuum and cold, to reinforce the reliefs). A uniform and very thin carbon film is then sprayed over the metallized surface to strengthen it and cover the areas not reached by the metal. This has produced a truly precise cast of the fractured surface of the sample: the replica; this is what is observed under the transmission electron microscope.Before observation, it will have to be detached from the sample by thawing, rinsed and finally placed on an electron microscopy grid.

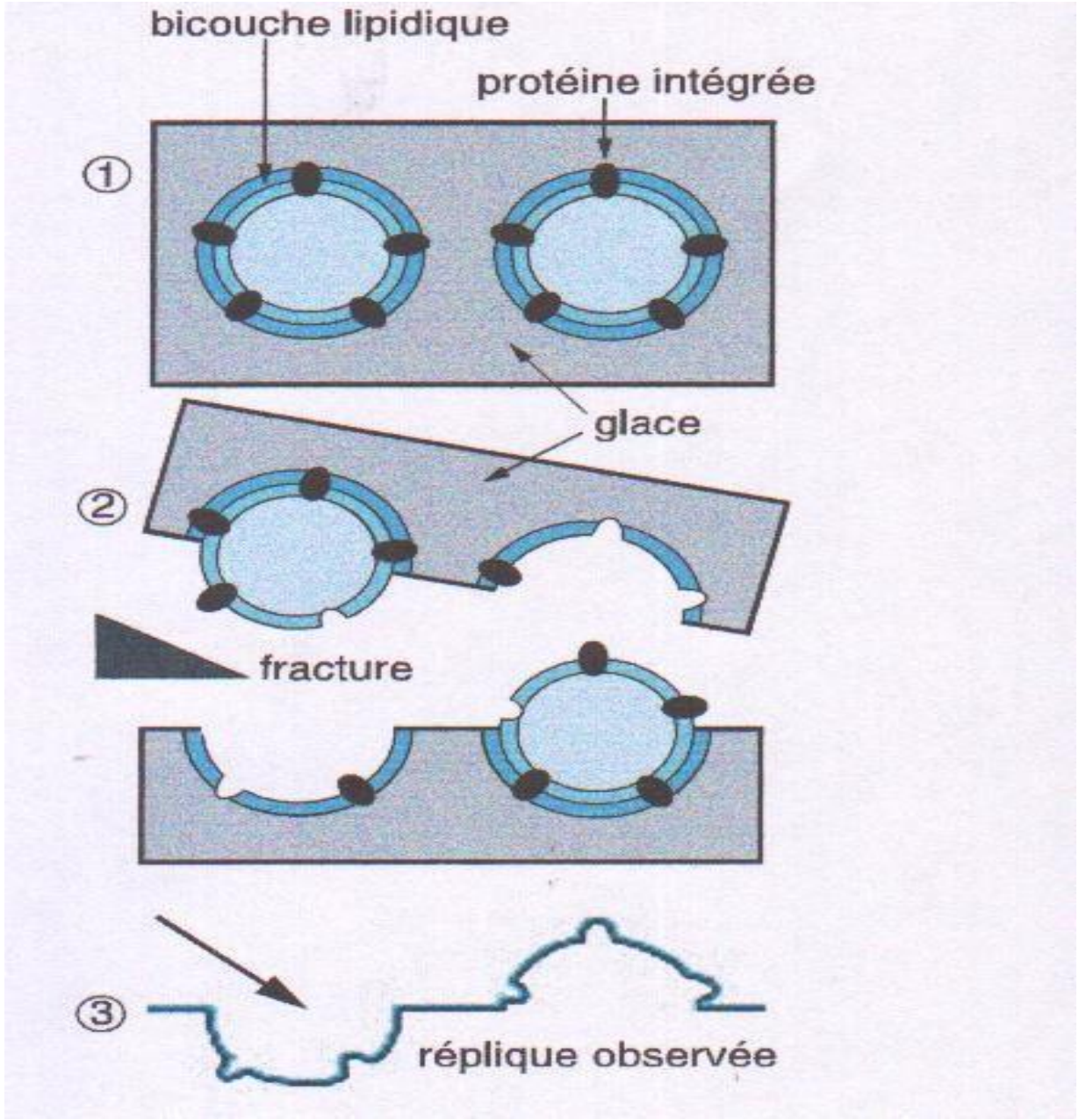


Figure 7: Principle of image formation during the freeze-fracture/freeze-etching protocol (1) Example of two proteoliposomes embedded in ice.(2) Ice fracture exposing either the concave part (left) or the convex part (right) of the vesicles. (3) Appearance of the metallic replica obtained after unidirectional shadowing (arrow) and carbon vaporization. The fracture reveals the upper surface or the imprint of the lower surface of the intrinsic proteins, depending on their degree of burial in the bilayer. The metallic replica of this surface thus presents "hollows" or "bumps", depending on the case**.**

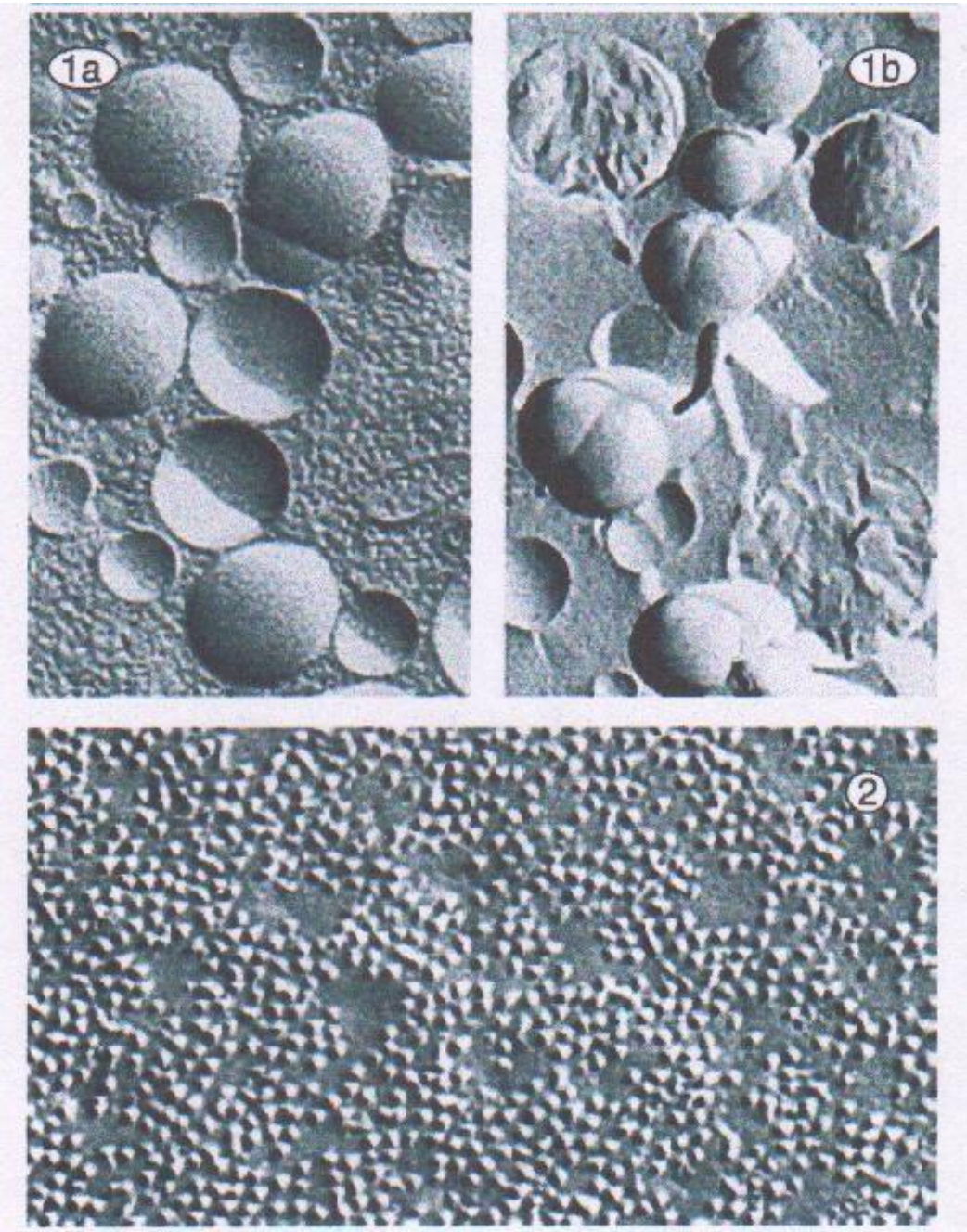


Figure 8.Image obtained by freeze-fracture

(1a) Appearance of liposomes containing proteins integrated into the bilayer (proteoliposomes) and having a rough surface. (1b) Appearance of smooth liposomes consisting solely of lipids. (2) Appearance of a biological membrane (here the cytoplasmic membrane of a Xenopus oocyte; x 75,000**)**

3- Freeze-fracture and freeze

-scalingThis technique is an evolution of the so-called metal fracturing technique developed ten years earlier. It is based first on the very rapid freezing of a very small biological sample (less than 1 mm3) in liquid nitrogen (–196°C). The crucial step in the protocol is the fracture, not the cutting, of the frozen sample, and this is done at very low temperature. This freeze-fracture releases an irregular surface across the sample, and it is this surface that will be observed. The sample surface is then etched by sublimating, under vacuum and at low temperature, a thin film of superficial ice; this has the effect of very slightly increasing the relief of the structures (freeze-scaling). Metallic shadowing is then carried out, under vacuum and cold, to reinforce the relief. A uniform and very thin carbon film is then sprayed onto the metallized surface to reinforce it and cover the areas not reached by the metal. This gives a truly precise cast of the fractured surface of the sample: the replica; this is what is observed under the transmission electron microscope. Before observation, it must be detached from the sample by thawing, rinsed and finally placed on an electron microscopy grid.

- **II. Histochemical methods**
- **Histochemistry**

is the study of the chemical composition of cells, various living tissues and cellular and tissue chemical reactions during metabolic processes.

Histochemical techniques allow to specifically recognize chemical groups or substances and to localize them in a precise manner. They offer the possibility to study and recognize the distribution of nucleic acids, lipids, carbohydrates, proteins in the cell, to localize enzymes.

So the histochemist seeks to localize a given substance in a histological structure.

II. Histochemical methods

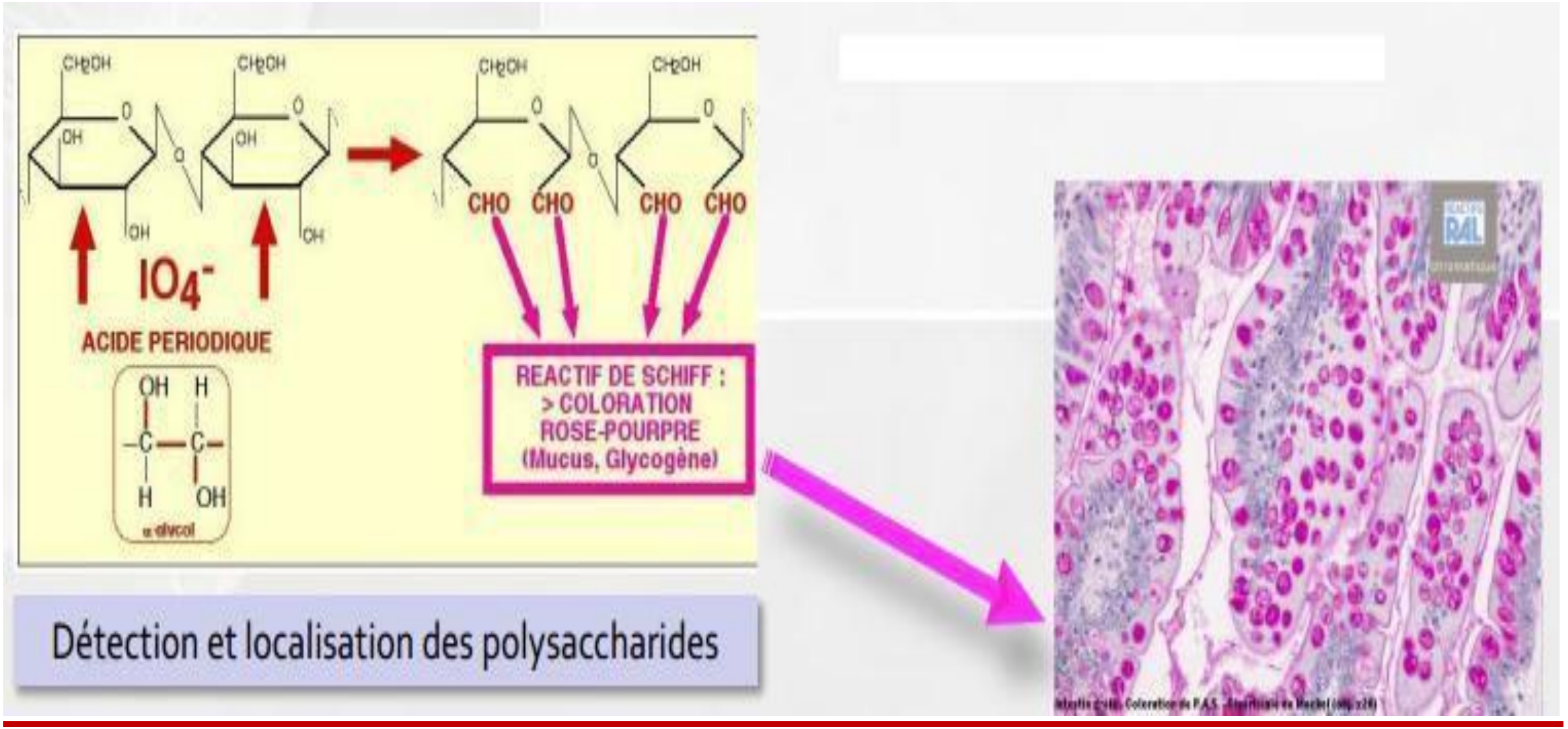
Histochemical techniques:

Histochemical techniques are based on biochemical reactions that allow in situ (= in tissues)

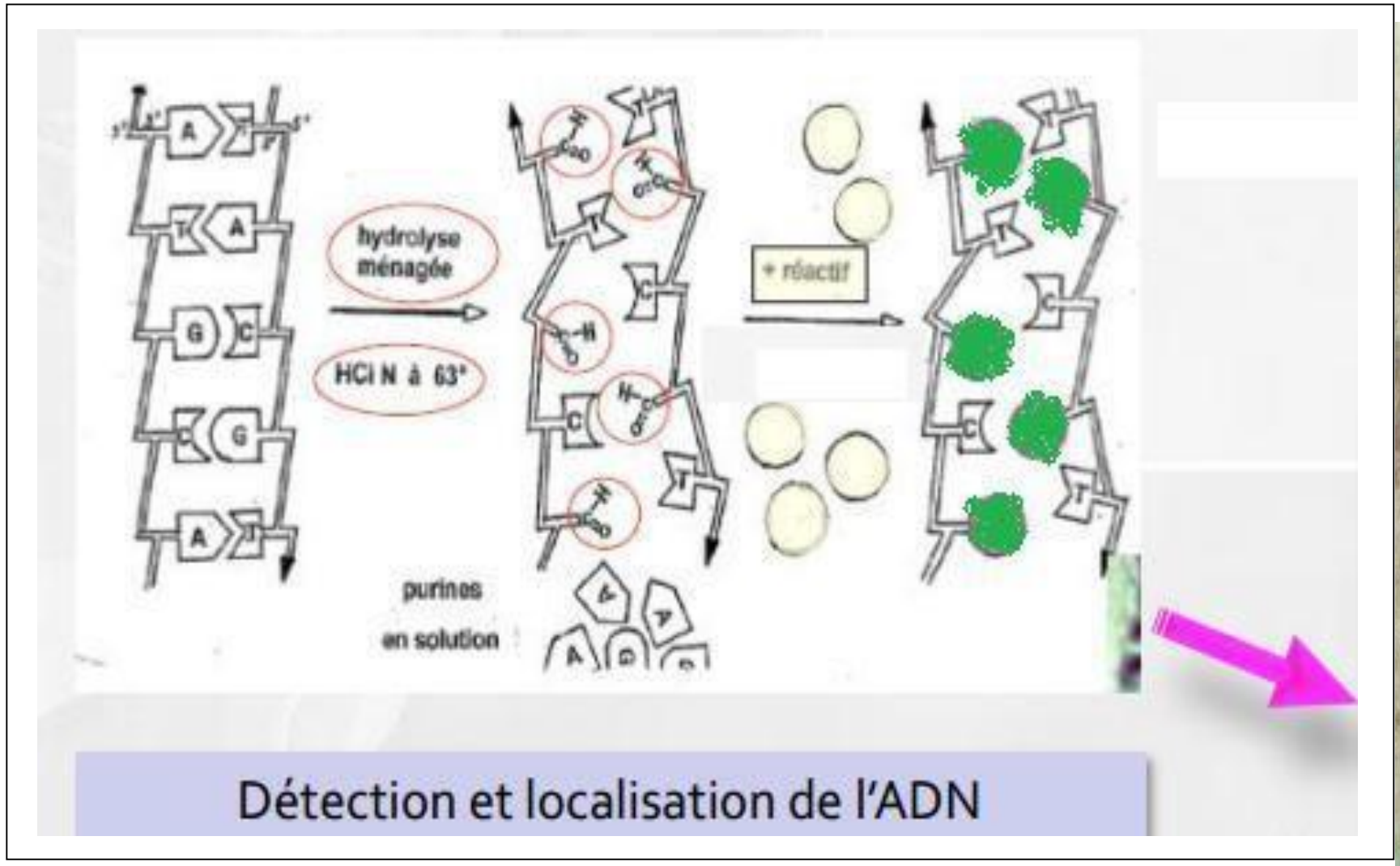
detection of different constituents (lipids, carbohydrates, proteins, nucleic acids, metals, etc.). Therefore, the histochemist seeks to locate a given substance in a histological structure.

II.1 Detection of carbohydrates Periodic acid (IO_4H) is an oxidant that cuts the 1-2 glycol, amino alcohol bonds of carbohydrates and glycoproteins and forms polyaldehydes. These aldehydes are then combined with Schiff's reagent (acid fuchsin) which gives a purple-red color with the structures containing the aldehydes.

Figure . Polysaccharide localization reaction



- **Nucleic Acid Localization:**
- Brachet Test
- This technique is used to localize nucleic acids within the cell. It is always performed using sections. This test combines:
- A mixed staining method (methyl green + pyronin red):
- **Methyl green > green DNA**
- **Pyronin red > red RNA**
- The use of enzymes that specifically hydrolyze either DNA or RNA, in the case of DNase and RNase, is necessary.



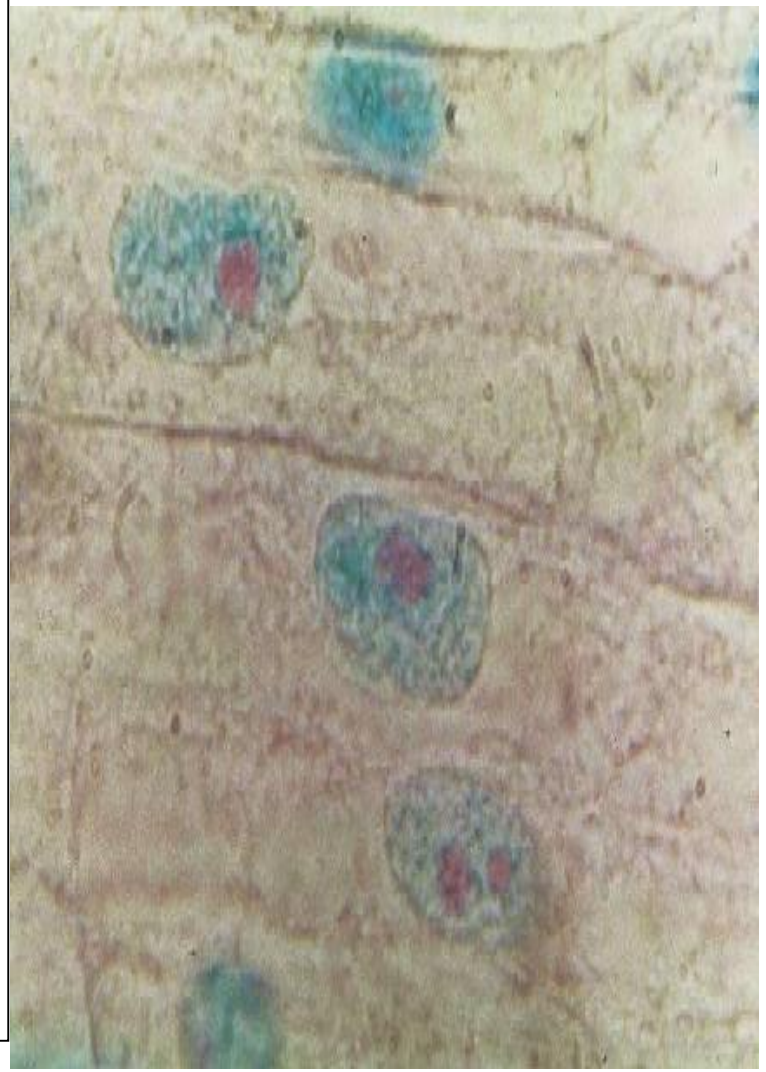


Figure . DNA localization reaction.

- **Highlighting lipids**

- **Oil Red:**

Oil Red O staining is based on the use of a lysochrome (a fat-soluble dye) and an azo dye used to visualize neutral triglycerides and lipids in tissue sections. It is much more intense, making it easier to visualize.

Oil Red O staining is performed on fresh or frozen samples. Paraffin-embedded tissues are not compatible with this staining because the section preparation and, especially, the use of solvents, remove lipids.This stain is primarily recommended for parathyroid gland pathologies containing acini and fat vacuoles.

- Sudan Black B

- staining is intended for the visualization of lipids.

is used for staining a wide variety of lipids such as phospholipids, sterols, and neutral triglycerides.Sudan Black B is not lipid-specific like other Sudan stains and can also be used for staining chromosomes, the Golgi apparatus, and leukocyte granules.

Highlighting micro-organisms Gram:

Widely used in medical bacteriology; it allows bacteria to be stained and distinguished by their ability to fix Gram+ gentian violet or Gram- fuchsin.

Immunological techniques (Ag antigen localization technique)

Precipitation methods: The precipitation phenomenon occurs when a soluble antigen is brought into contact with the corresponding antibody. This reaction occurs either in a liquid medium or in a gel medium. The reaction time varies from less than an hour to several days. The reading can be done either with the naked eye or with devices such as the nephelometer or the turbidimeter.

Agglutination methods: Agglutination occurs when a particulate antigen (bacteria, red blood cells, etc.) is brought into contact with the corresponding antibody. This reaction is rapid (a few minutes) and visible to the naked eye.

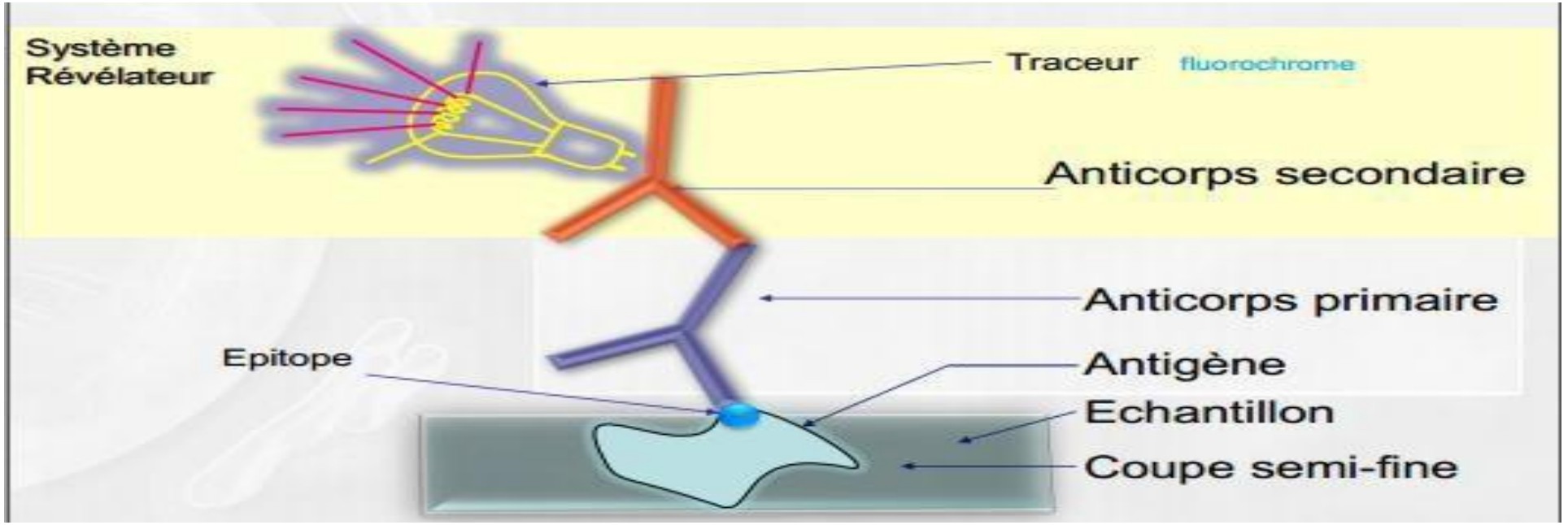
Methods using a marker:

These are the most sensitive methods (ng/mL and pg/mL). Depending on the marker used, we can distinguish 3 types of technique:

Immunofluorescence (the antigen or antibody is labeled with a fluorochrome): The time to perform an immunofluorescence technique is less than 2 hours. Reading requires the use of a fluorescence microscope or a flow cytometer.

Immunoenzymology (from ELISA 'Enzyme Linked Immuno Sorbent Assay'): In this reaction, the antigen or antibody is labeled with an enzyme, which transforms a colorless substrate into a colored product. The time to perform varies from less than an hour to several hours. Reading is carried out using a spectrophotometer.

Radioimmunology: Also known as RIA (Radio-Immuno-Assay) based on the use of a radioactive marker.



- **VI. Enzymological techniques**

Histoenzymology is a specialization of histology and in particular of histochemistry. Its aim is to indirectly detect the specific activities of certain enzymes by methods that require histological stains and specific substrates.

1 The ELISA technique:

2.1.1 Principle: The ELISA technique (Enzyme Linked Immunosorbent Assay) is a detection technique which allows visualization of an antigen-antibody reaction thanks to a colored reaction produced by the action on a substrate of an enzyme previously fixed to the antibody.

- **The indirect ELISA test**
- **the first step is called:**

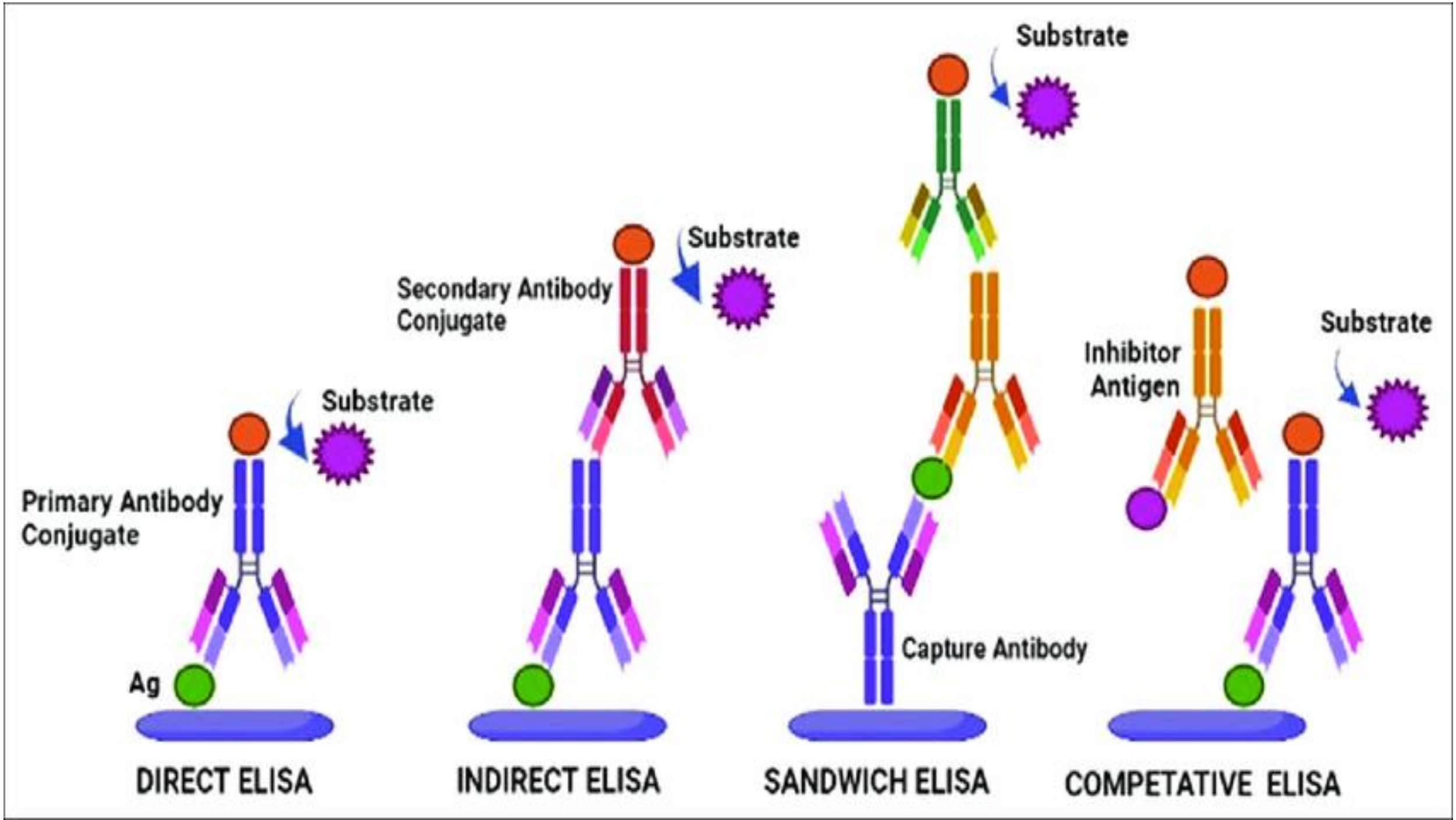
Coating" the antigen: This consists of incubating the antigen solution specific to the desired antibody in wells. The antigen is fixed to the bottom of the wells electrostatically. The plates are incubated at 4°C overnight. The wells are then washed to remove excess antigens with washing buffer

second step consists of

Fixing the antibody to be assayed: The antibody solution to be assayed is incubated at 37°C in the wells for approximately 30 minutes to 2 hours. The antibodies bind specifically to the antigen. The wells are then washed to remove excess antibodies to be assayed with washing buffer.

third step is to

Fixing the detection antibody: The detection antibody solution is incubated at 37°C in the wells for approximately 30 minutes to 2 hours. The detection antibodies bind specifically to the antibodies to be assayed. The wells are then washed to remove excess detection antibodies with wash buffer. Note that the detection antibodies are coupled to an enzyme which in the presence of its substrate transforms it into a detectable and measurable reaction product thanks to the appearance of a color.


Substrate
Secondary Antibody
Conjugate
Substrate
Substrate
Primary Antibody
Conjugate
Inhibitor
Antigen
Substrate
Ag
Capture Antibody
DIRECT ELISA
INDIRECT ELISA
SANDWICH ELISA
COMPETATIVE ELISA

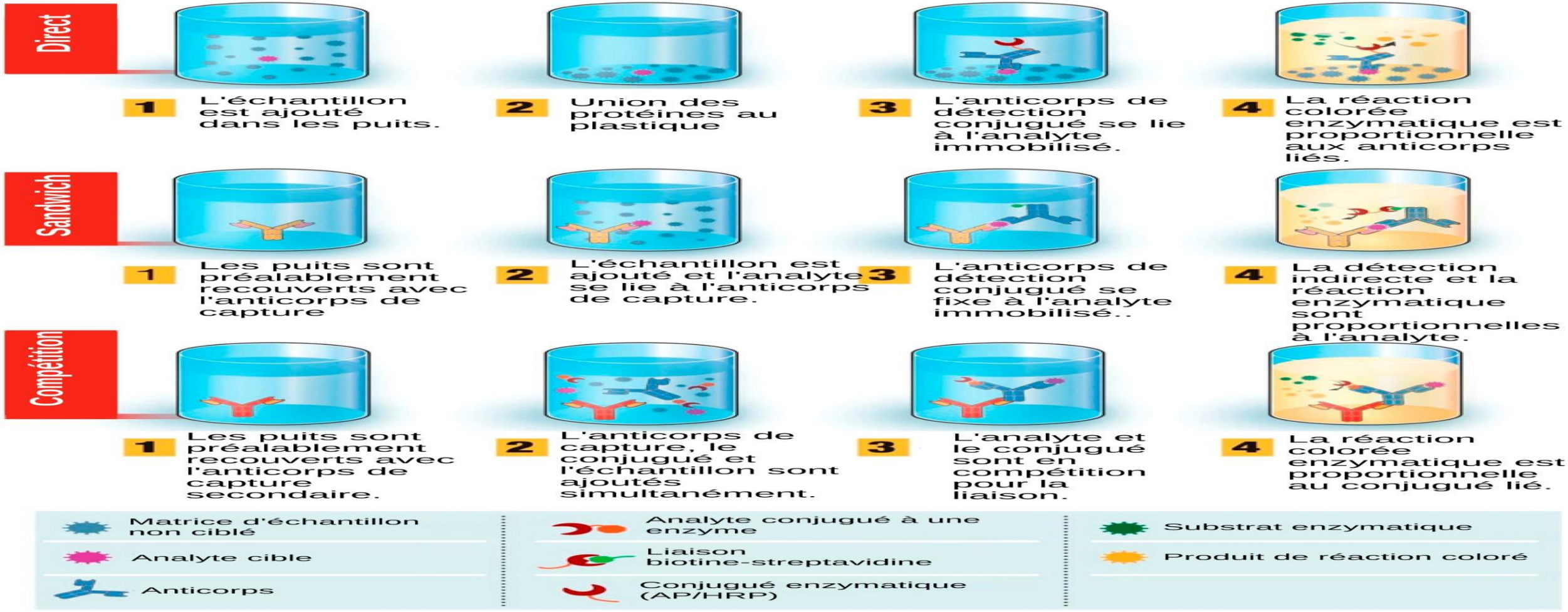


Figure 1: Overview of the enzyme-linked immunosorbent assay (ELISA)-based diagnostic test. The ELISA test can be presented in different formats depending on the differences in antigen immobilization and antibody labeling. In the direct ELISA test, virus antigens bound to a plastic solid phase are detected by the addition of a conjugated antibody. In the sandwich ELISA test, the capture antibody binds to the plastic solid phase. The sample antigens bind to the capture antibody and are then detected by a second enzyme-labeled antibody. In the competition ELISA test, the viral antigen in the sample is pre-incubated with the primary antibody and then added to a well coated with a secondary antibody with an enzyme-conjugated antigen that competes with the sample antigen for binding to the primary antibody. The more viral antigen present in the sample, the less the conjugated antigen will bind and the weaker the signal will be. Source: Adapted from Ghaffari et al. 2020.

The fourth step consists of

revealing the bound antibodies:

A developer solution containing the enzyme substrate is incubated at room temperature and in the dark for 10 minutes. The appearance of a color in the substrate indicates the presence of the antibody to be measured. The intensity of this color is proportional to the amount of enzyme present and therefore to the concentration of the desired antibody.Application example: Identification of antibodies to the envelope and body proteins of the human immunodeficiency virus in patient serum.

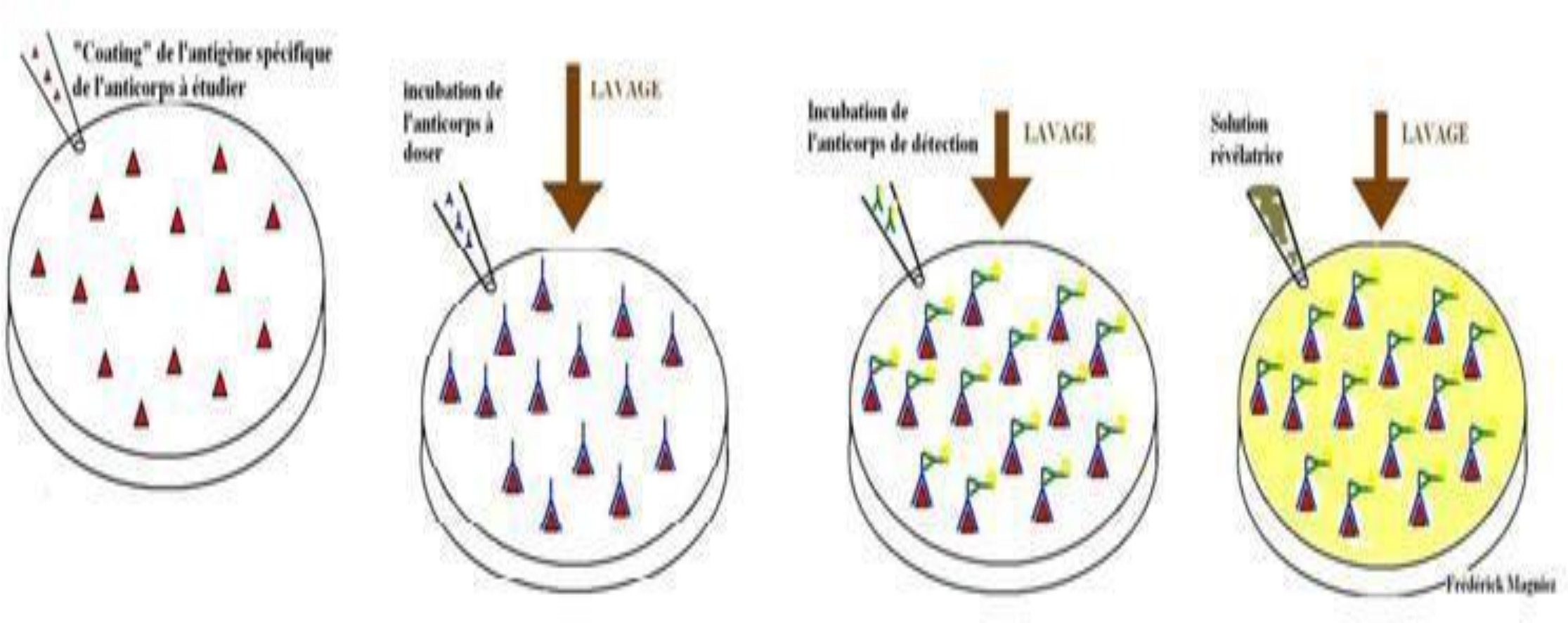


Figure: The indirect ELISA method

- **Direct DAS ELISA or Direct Double Antibody Sandwich ELIS**A
- The use of the DAS ELISA requires two monoclonal antibodies that recognize different epitopes on the antigen.

The first step involves attaching the capture antibody to the substrate. The solution is incubated at 37°C for 2 hours, then washed, or overnight at 4°C, then washed.

In the second step, the sample containing the antigen to be identified is added and incubated at 37°C for 2 hours, then washed, or overnight at 4°C, then washed.

In the third step, the enzyme-labeled detection antibody is attached to the desired antigen. To do this, the antibody solution is added to the wells and the whole mixture is incubated at 37°C for 2 hours.

In the final step, a developer solution containing the substrate for the enzyme is added and incubated for 30 to 120 minutes. The resulting reaction product is soluble and colored. The intensity of this color can be measured using a photometer.