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Cellular engineering

Intended for first-year masters students Biological sciences
Biotechnology and health speciality
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Preface

This handout is the written support for the module “Cellular Engineering” intended for students of the Master 1 specialty biotechnology and health. The courses in this module aim to acquire the theoretical and practical foundations of cell engineering applied discipline which is devoted to the modification of genetic sequences in living cells, It brings together in a condensed way the essential information on the methods of studying cells and molecules as well as the most used analytical techniques on the cell in quantitative and qualitative analysis.

This document is designed according to the official outline prescribed by the Ministry of Higher Education and Scientific Research. These courses are organized in seven chapters:

1. First, the fractionation techniques are presented. In addition, the different principles are discussed and the materials used;
2. In the second chapters, immunomarking techniques were presented.
3. The third chapter is devoted to cell observation techniques: optical and electron microscopy, their principles, types, sample preparation and their domains of application.
4. The fourth chapter presents the flow cytometry methods, Tricellular by quoting the different types, their principles and the materials used.
5. The fifth chapter presents cell cultures: types, conservation methods and experimental conditions.
6. The sixth chapter deals with toxicity and viability tests performed on cells, their advantages and disadvantages
7. The last chapter summarizes the cell, tissue and animal models while discussing the types used, appropriate choices and ethical laws.

Cellular Fractionation

Cellfractionation

Cell fractionation is a technique for isolating or purifying organelles while preserving their individual functions. This technique involves separating the different components of the cell by breaking down the plasma membrane to analyze their structure and function.

Filtration

Filtration is a separation process that separates the components of a mixture that has both a liquid phase and a solid phase through a porous medium. It is an operation in which the solid particles remain on the filter, and a homogeneous mixture is recovered. The use of a filter allows the retention of particles in the heterogeneous mixture that are larger than the holes in the filter (porosity). The liquid that has undergone filtration is called the filtrate, and what the filter retains is called the residue.

Principle

Filtration is essentially a sieving process, i.e., a separation based on the diameter of solid particles of different sizes. A pressure difference forces the liquid to pass through the filter while the solid particles are retained on the surface.

Moreover, filtration is often accompanied by additional phenomena, which are:

a. **Clogging**: This is caused by particles that deposit on the surface of the filter but mainly by their penetration into the interstices of the filtering material. This clogging completely changes the porosity and slows down filtration.

b. **Adsorption**: The filtering material has a certain electrical charge. Thus, some substances may be retained even though their size allows them to pass through the filter pores.

1.1.1.2. Materials

a. Filters: There are two types of filters: depth filters (thick or depth) and surface filters (membrane).

- **Depth Filters**: These are made of fibrous substances (paper, asbestos, cellulose, cotton, fiberglass, etc.) or agglomerated substances (fritted glass, sand, charcoal, etc.). The efficiency of a depth filter increases with its thickness, but it decreases when the pressure applied to the filter increases.

The materials used in depth filters include:

- **Classic filter paper**: There are various qualities of paper that differ in their shape (rectangular, circular, pleated sheets, etc.), texture (loose, fine), porosity, and purity (crude, purified, ashless, etc.).
- **Textiles** :Gauze, cotton, wool, nylon, etc.
- **Fibers**:Fiberglass, asbestos.
- **Clays and porcelain**
- **Fritted material**: Fritted glass is obtained by compressing glass beads at a controlled temperature, resulting in variable porosities and shapes.
- **Surface Filters**: The most commonly used are Millipore membranes. These are very thin sheets made of cellulose polymer with a large number of precisely calibrated pores. The pore sizes vary depending on the filter, from 0.025 µm to 8 µm.

b. Funnels

Funnels are conical instruments designed to hold filtering material. There are two types:

- **Ordinary funnels**: Made of glass, porcelain, or polycarbonate,
- **Special funnels**: These can be made of glass or porcelain with a perforated bottom plate, on which a filter paper disc is placed that exactly, covers the bottom. This category includes

Buchner funnels, used for filtering relatively large quantities of solids, and Hirsch funnels, used for recovering small quantities of solids (**Figure 1**).

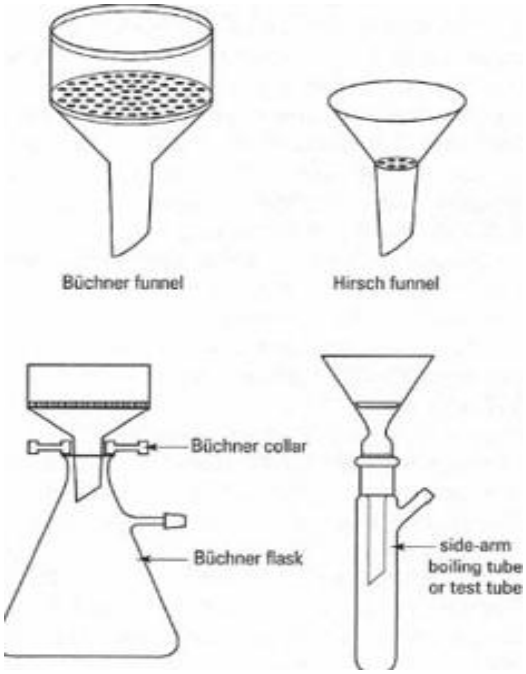


Figure 1: Filter funnel.

Laboratory filtration methods

a. Gravity filtration

This method uses a filter and a container to hold the filter. The simplest device for this method is a single funnel with a paper filter mounted on an Erlenmeyer to collect the filtrate (**Figure 2**). The pressure difference is created by the height of the liquid on the filter. For this method, two elements are required:

The shape of the container should encourage the flow of liquid through the filter. It is important to avoid that the funnel creates an obstacle for the flow of liquid. This gives the shape of the funnel some importance. The angle of opening of the funnel should be close to 60°.

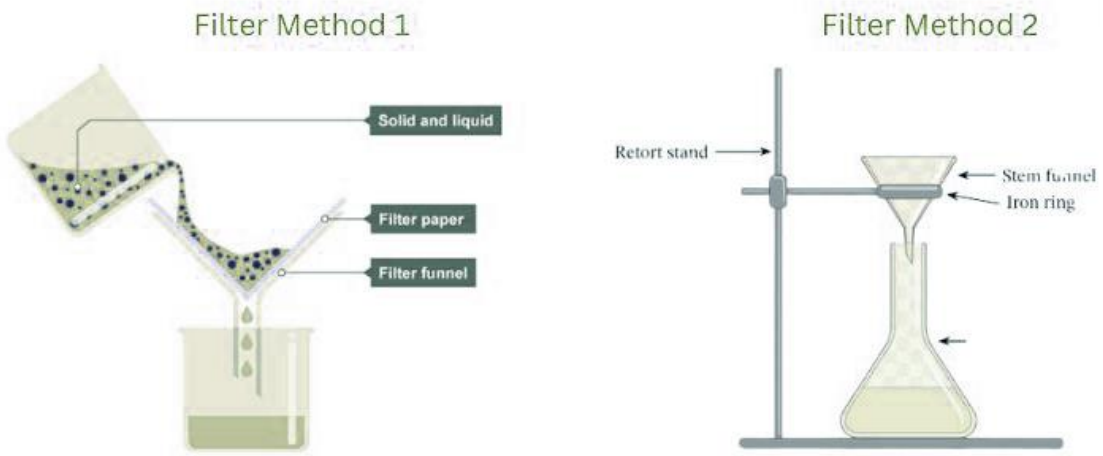


Figure 2 : Gravity filtration.

This type of filtration has some disadvantages:

• Fairly slow filtration,

• Difficulty in recovering the isolated solid phase, especially when it is not abundant (it is dispersed over a fairly large area of filter paper, to which the grains adhere),
• Incomplete separation: the solid retains a significant amount of liquid.

b. Vacuum filtration

The filtration speed is increased by causing a downstream depression of the filter material. To achieve vacuum filtration, special funnels are used that are fitted on a "suction flask", in which a depression is created. The funnel is usually fitted to the flask by means of a rubber cone, which will "stick" to the flask and the funnel when the depression is established (**Figure 3**).

Figure 3. Vacuum filtration.

c. Filtration under pressure

Filtration under pressure prevents foaming and evaporation of the solvent. In the laboratory, sterilizing microfiltration using the Millipore device is a pressure filtration. This device consists of two plastic parts, which are screwed together, enclosing a membrane filter (**Figure 4**).

Figure 4. Filtration under pressure.

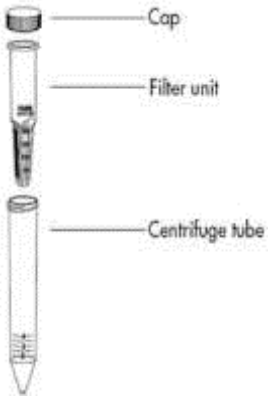
d. Ultrafiltration

Ultrafiltration covers a very wide range of molecular weight ranging from 500 to 1,000 tons. It is a membrane with such low porosity that it can retain proteins and nucleic acids. These filters may have pores as small as 25 nm that can hold most proteins. Ultrafiltration allows:

• Concentrate solutions of macromolecules.
• Remove most small molecular contaminants (salt, carbohydrate...).

Membranes with a porosity allowing certain substances to pass (salts, small molecules, small proteins) are used. These membranes are most often sold as filtration units (**Figure 5**), which consist of a tube with the lower part separated from the upper part by the membrane. The proteins are deposited in the upper part of the tube, and rest on the membrane. The tube is then centrifuged to force the liquid and small molecules through it under the centrifugal force

Figure 5. Ultrafiltration unit.



Several applications can be cited:

- **Clarification:**obtaining a liquid free of solid particles.
- **Sterilisation:**many biological molecules are heat-sensitive and cannot be sterilised in an autoclave. The solution to this problem is cold sterilisation. The surface filters we use have pores 0.45µm or 0.22µm in diameter. Bacteria are all retained by a 0.45µm filter. Those with a pore size of 0.22µm.
- **Desalting:**to remove the salts used during a purification stage, for example. Concentration: concentrating a diluted sample.

Centrifugation

The word centrifugation is derived from the verb "centrifuger" which comes from the Latin fugere meaning "to flee" and "centre", to which is added the suffix -action indicating an action.

Centrifugation is a process for separating compounds in a mixture on the basis of their difference in density by subjecting them to centrifugal force. The mixture to be separated may consist of two liquid phases or solid particles suspended in a fluid.

A preparation can thus be split into a sediment (or "pellet"), made up of material more or less solidly packed in the bottom of the centrifuge tube, and a supernatant, which is the residual liquid above the sediment (**Figure 6**).

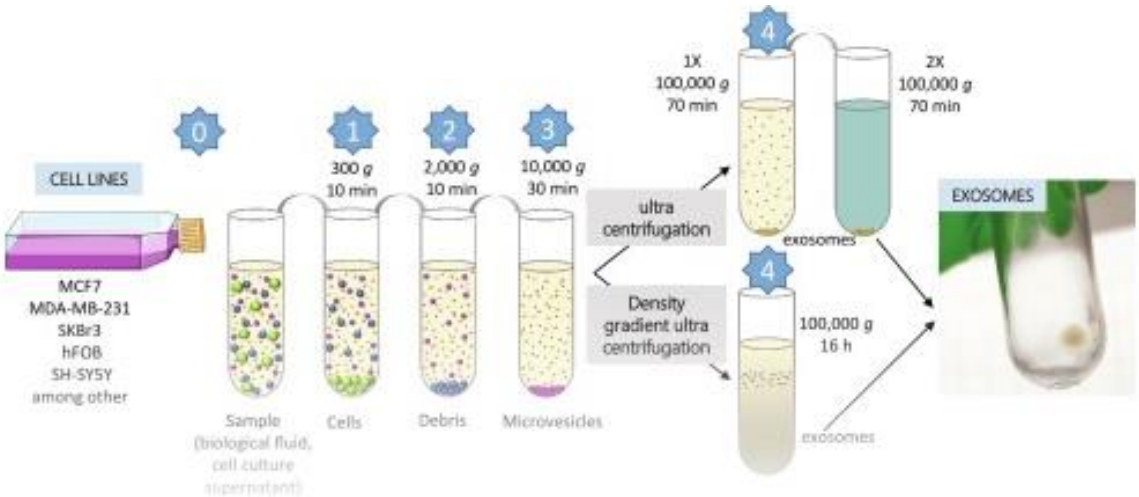


Figure 6. Centrifugation.

Principle

All the constituents contained in a solution are subject to:

- Gravity: force from top to bottom,
- Archimedes' buoyancy: force from bottom to top,

Rotating the sample creates a new force, the centrifugal force, which is an outward acceleration that causes it to settle or rise (**Figure 7**).

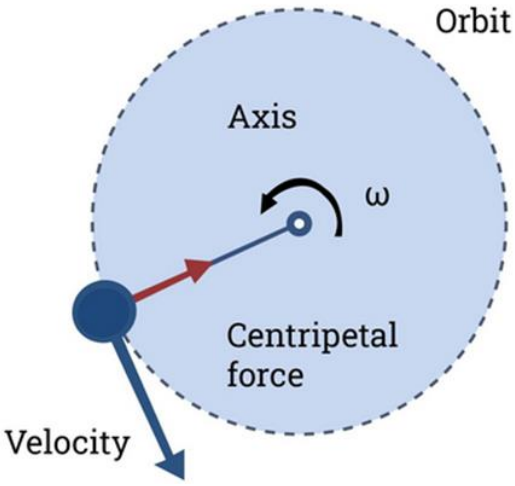


Figure7. Centrifuge working principle.

Calculating the gravitational force

In a centrifuge, you need to know the relative centrifugal force (relative gravitational force, RGF) in "g" or the acceleration g. However, for a given speed of rotation, each rotor has a different GRF because the radius of rotation is different. You therefore need to be able to convert the rotational speed (RPM, revolutions per minute) into FGR.

The mathematical formula for conversion (Get rpm): FGR(g)= 1.119.10-5xrpm2xr

Where r is the radius of rotation of the rotor (in cm) and RPM (revolutions per minute) expresses the speed of rotation.

Types of centrifugations

a. Differential centrifugation

In a first centrifugation at low acceleration, the most massive elements will sediment and form a pellet at the bottom of the tube. All the other elements remain in the liquid fraction known as the supernatant. The supernatant and the pellet are then recovered separately, which means that the components have been separated. If necessary, a second centrifugation cycle can be repeated using the previous supernatant, but at a higher speed. In this way, the various components are gradually separated, ending with the smallest elements that have the least difference in density with the solvent (**Figure 8**).

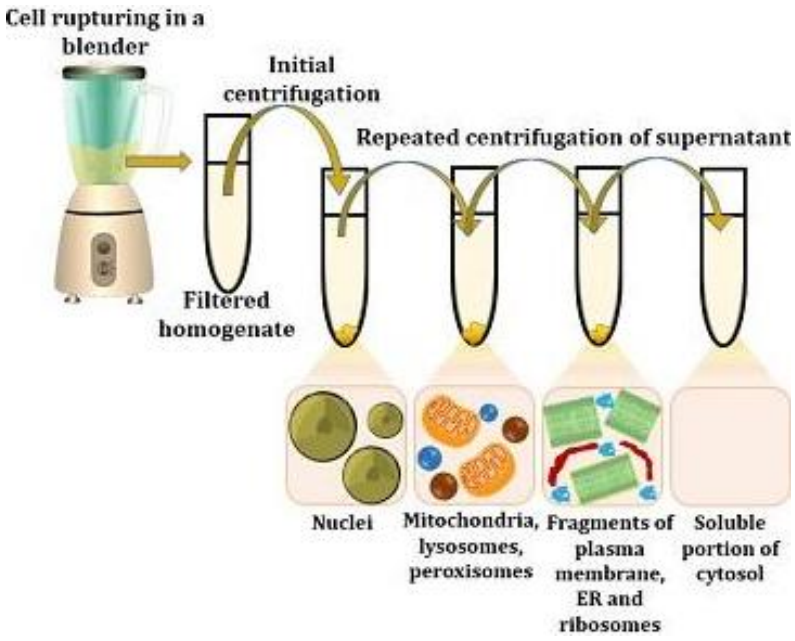


Figure 8: Differential centrifugation.

b. Density gradient centrifugation

In density gradient centrifugation, the various constituents reach a position from which they will not move, as they are in equilibrium. Equilibrium is reached when the density of a particle is equal to the density of the solvent, which means that the gravitational force is equal to Archimedes' buoyancy. We will therefore use a solvent whose density will vary according to the position in the tube, allowing the different constituents to reach the zone of equivalent density: this is known as a gradient.

Separation methods can be enhanced or refined by centrifuging in a concentration gradient. In fact, one of the factors influencing the speed of sedimentation.

The difference in density between the particle and the solvent can be adjusted to modulate this rate by creating a density gradient.

- If the particle's density is greater than that of the medium, it will sediment. The greater the densitydifference, the faster the sedimentation.
- If there is no density difference, there will be no sedimentation, regardless of the acceleration.
- If the particle is less dense than the medium, it will rise in the tube until it reaches a density level equal to its own or, if applicable, until it floats to the surface.

c. Isopycnic centrifugation

Molecules subjected to gradient centrifugation will separate according to their density, not their mass. The preparation to be analyzed is mixed with the gradient. Each type of particle will sediment until it reaches the concentration corresponding to its density, where it will become immobilized.

d. Zonal centrifugation or separation

Centrifugation can also be used for analytical purposes to separate particles or molecules based on their size. In these applications, as the particles are homogeneous, they all have the same density and differ only in size. The gradient ensures that heavier particles sediment faster than lighter ones without ever reaching the bottom of the centrifuge tube. At the start of this centrifugation, the sample is deposited on a liquid column forming a density gradient.

Rotors

There are two main types of rotors: fixed-angle and swinging-bucket.

a. Fixed angle rotors

Fixed-angle rotors are made of metal blocks (aluminum, titanium) with wells drilled inside and tilted at a certain angle to the horizontal, generally between 15° and 35°, depending on the model. Centrifuge tubes are placed in these wells. Since these rotors are relatively compact, they can be spun quickly due to their relatively short radius. Particles will mostly sediment along the tube wall. Moreover, they tend to accumulate on the sides at the bottom of the centrifuge tube. For certain types of particles, this causes friction that they cannot withstand and may break. However, most medium- and high-speed centrifugations use this type of rotor.

b. Swinging bucket rotors

Swinging-bucket rotors reorient themselves during centrifugation. The buckets are arranged on hooks or a swinging system. When the rotor starts spinning, the buckets (and the tubes they contain), under the effect of centrifugal force, reorient themselves and move into a horizontal position. Particles can then sediment directly to the bottom of the tube without ever hitting the tube walls. They accumulate at the bottom of the centrifuge tube. The main disadvantage of this type of rotor is that it cannot reach very high speeds compared to the other type. The horizontal position of the buckets significantly increases the rotor's radius, making it more difficult to achieve high rotational speeds. This type of rotor is used in both discontinuous and continuous gradient centrifugations.

c. Centrifuges

A centrifuge is a machine equipped with a rotating axis enclosed in a chamber. Except for benchtop centrifuges, where the rotation speed and typical usage time are relatively limited, it is necessary to prevent sample heating. To achieve this, the chamber is refrigerated and often subjected to a strong vacuum to reduce friction.

A range of devices has been developed based on experimental needs, particularly regarding the required accelerations, the volumes of material to be centrifuged, working temperature, etc.:

- Tabletop centrifuge
- Floorcentrifuge
- Ultracentrifuge
- Microcentrifuge

Dialysis

It often happens that a preparation of macromolecules contains various products that need to be removed. These products, such as salts, carbohydrates, or other small molecules, were present in the initial preparation or were introduced during a purification step (e.g., extraction or precipitation). A simple way to eliminate these small molecules is dialysis.

Principle

Dialysis is based on the phenomenon of diffusion through a semi-permeable membrane. Two mechanisms are involved in this process:

- Diffusible molecules will pass through the membrane according to the concentration gradient, resulting in a net movement of molecules from the more concentrated side to the less concentrated side.
- At equilibrium, the concentrations of each diffusible species will be equal on both sides (**Figure 9**).

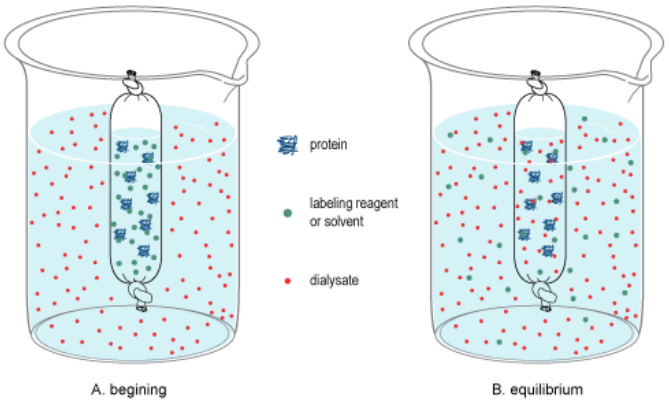


Figure 9. Dialysis principle.

Factors influencing dialysis

Several factors related to the characteristics of the dialysis membrane, the counter dialysis solution, and the solution to be dialyzed can affect the proper course of dialysis:

- **Electrical charge of the dialysis membrane**: The membrane should not be electrically charged to minimize repulsion phenomena due to identical charges or adsorption of the product to be dialyzed onto the membrane.
- **Temperature**: The diffusion rate of a substance across the membrane increases with rising temperature, which amplifies the agitation of molecules and thus increases the probability of a given molecule passing through the membrane.
- **Membrane surface area to volume ratio of the solution to be dialyzed**: For a given volume of solution to be dialyzed, the rate increases with the membrane surface area.
- **Presence of chemical substances modifying the dialysis rate by inhibiting or increasing it**: Adrenocorticotropic hormone from the pituitary gland is dialyzed against distilled water. If the water is replaced with a 0.15M ammonium solution, dialysis is almost inhibited due to the formation of non-dialyzable aggregates, while the addition of urea increases the dialysis rate.

Dialysis materials

a. Membranes

There are several materials used to manufacture dialysis membranes, with the most commonly used being cellulose esters or other cellulose derivatives (Figure 10). Membranes can be produced with various levels of porosity, ranging from 100 to one million daltons(in terms of protein size), and are easy to control during the manufacturing process. Some manufacturers have developed micro-dialysis systems for handling volumes as small as 250 µl.

Figure 10. Dialysis membrane.

c. A **dialyzer** is designed using a membrane with a large surface area and thin thickness. Additionally, the concentration of the diffusing substance in the counter dialysis liquid is kept at zero, either by using a large volume of this liquid or by constantly renewing it. There are several types of dialyzers; the first dialyzer constructed was the Graham dialyzer, followed by the Monod dialyzer.

d. Dialysis Techniques

- **Preparation of dialysis membranes:** dialysis membranes contain glycerin, sulfur compounds, and heavy metals that must be removed beforehand by soaking. The usual technique involves placing the membranes for one hour in a mixture of equal volumes of ethanol and water, then for one hour in a 10mM sodium bicarbonate solution. Next, they are soaked for one hour in a diluted EDTA (Ethylene Diamine Tetra Acetate: a chelating agent that binds heavy metals) solution, followed by two hours in distilled water. The prepared membrane is then stored at 4°C for two to three days in water.
- **Preparation of dialysis tubing**:the dialysis tubing should be tied at both ends with a single simple knot. The dialysis should be tested with glass beads inside the tubing. This prevents it from floating on the surface of the counter dialysis liquid, as this would restrict the contact surface, thereby reducing the dialysis rate. Proper agitation of the counter dialysis liquid should be ensured to prevent the formation of a concentration gradient of diffusible substances in the container holding the counter dialysis liquid.

To accelerate the dialysis process or prevent the salts from equilibrating their concentration across the membrane:

- The dialysis buffer should be changed frequently.
- A much larger volume of dialysis buffer than the protein solution should be used.
- The dialysis tubing should be agitated.

Applications of dialysis

- Remove diffusible productscontained in protein solutions.
- Concentrate a protein solution by either placing the dialysis tubing containing the solution in an air stream or placing the tubing in powdered polyethylene glycol (PEG). The water leaves the tubing to solubilize the PEG, which is non-diffusible.
- Electrodialysis allows desalting samples before performing paper chromatography.

Precipitation

The cell contains a large number of proteins, among which the one of interest is likely a very minor component. It is possible to remove the majority of unwanted proteins. One of the quickest ways to do this is to precipitate them.

1.1.4.1. Types of precipitation

a. Total Precipitation

As the name suggests, total protein precipitation methods aim to eliminate all proteins from a solution and irreversibly denature the proteins. Total precipitation is used when one wants to separate proteins from other small contaminant molecules, such as amino acids, sugars, etc., or sometimes from other macromolecules. This approach is therefore incompatible with procedures where one wants to recover an intact and functional protein. Severalmethods of total precipitation are used:

- **Ethanol or Acetone Precipitation**: proteins can easily be precipitated in the presence of 80% ethanol (EtOH) by keeping them at -20°C for a few hours or by bringing the mixture to a boil for a few minutes. Centrifugation can then be used to sediment the precipitated proteins.

Residual ethanol can be removed by extracting the resuspended proteins in an aqueous medium with chloroform.

- **Phenol precipitation**: A method has been developed specifically to extract small amounts of proteins using phenol. The proteins denature, and many become soluble in phenol or aggregate at the phenol-water interface, unlike small molecules and nucleic acids, which remain soluble in the aqueous phase. It should be noted that this method is often used for the opposite purpose: to recover nucleic acids freed from proteins.
- **Alkaline or acidic media**: proteins are often only soluble within a narrow pH range. Thus, strongly acidifying or alkalizing a solution renders the proteins insoluble. Even moderate concentrations of acids can induce protein denaturation and precipitation.
- **Trichloroacetic acid precipitation (TCA)**: this approach involves adding TCA to the protein solution to achieve a final concentration of about 5-10% acid. To prevent protein degradation, all work should be done at 2-4°C. An exposure of 10 minutes is generally sufficient. Precipitated proteins can then be separated by low-speed centrifugation. Residual TCA can be removed by several washes with ether. The resultingproteinsedimentisverydifficult to redissolve.
- **Perchloric acid precipitation (PCA)**: Another similar acid precipitation method is using perchloric acid. Final concentrations of 5-7% are usually sufficient. One advantage of PCA over TCA is that it can be precipitated with KOH because potassium perchlorate is practically insoluble at 0-4°C. This is a much simpler approach than ether washes to remove excess PCA from proteins.
- **Zinc sulfate or sodium tungstate precipitation**: zinc sulfate (or cadmium sulfate) in the presence of a strong base (NaOH or Ba(OH) is an effective precipitant. However, this technique is somewhat complex, especially when using Ba(OH), which must be kept away from air. Another similar method uses sodium tungstate in the presence of a strong acid (H_2SO_4).

b. **Differential precipitation**

Differential precipitation, or fractional precipitation, is much milder than total precipitation. Differential precipitation does not denature proteins. This technique uses the differential solubility of proteins. Since each protein is more or less soluble in solution depending on its composition, several can be separated based on their tendency to precipitate more or less quickly when the ionic strength of the solution is changed.

1.1.4.2. Applications

- Separation of proteins from other contaminant molecules;
- A very common application of total protein precipitation is the separation of labeled proteins from radioactive amino acids that were used as precursors for their metabolic labeling.
- Protein purification one of the initial steps in purification procedures is often differential precipitation with ammonium sulfate. This relatively non-specific method is well-suited for the large volumes often obtained at the beginning of the purification process.

Immunomarquage

Definition

Classical radioimmunology is based on the principle of competitive binding between a certain quantity of antibody specific to a given antigen and the same antigen previously labelled with a radio isotope. A scintillator is then added to amplify the signal. An antibody-antigen complex is formed according to the equation: AC +Ag* = [Ac Ag*]. The antigen to be measured competes with the labelled antigen for binding to the antibody. The bound fraction, which decreases exponentially with the concentration of Ag to be assayed, is measured. So first, a calibration curve is made with Ag solutions of known concentrations and then the concentration of Ag contained in the serum is determined using the calibration curve **(Figure 11)**.

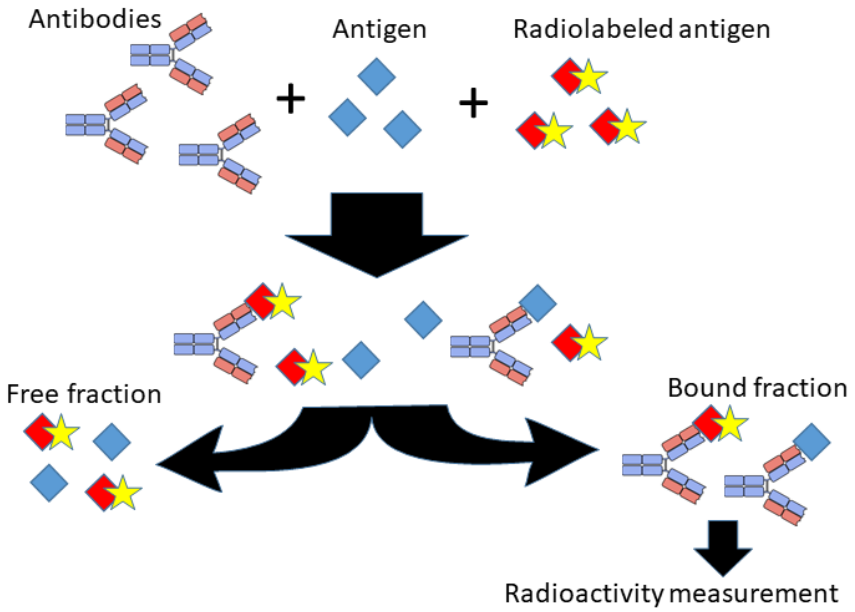


Figure 11. Radioimmunoassay.

Radioimmunoassay techniques

A method for measuring substances in very low concentrations using radioisotopes. The instability of radioisotopes means that, depending on the period or half-life corresponding to the disintegration of half the atoms present, traceable compounds are available for diagnostic and therapeutic purposes.The principle is based on competition according to the law of mass action between a cold antigen (Ag°) to be identified or quantified and a radiolabelled antigen (Ag*) in the presence of Ac. A biological liquid containing a fixed and very small quantity of labelled antigen (Ag*) is added to a medium containing an unknown quantity of cold antigen (Ag°) to be measured and a constant quantity of antibody.

2.2.1. Types

Radioimmunoassay by competition/ RIA: Radio Immuno Assay. Competition, antibody deficiency method, inverse proportional method (**Figure 12**).

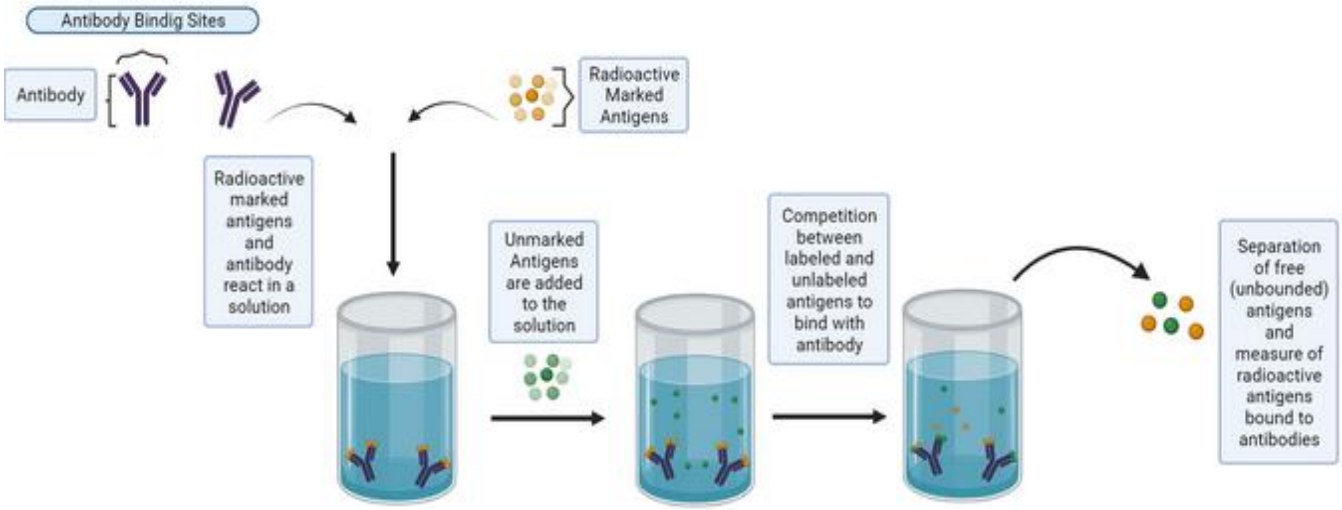


Figure 12. RIA (Radio ImmunoAssay).

Sandwich radioimmunoassay Immuno Radio Metric Assay/IRMA. Radio immunoassay by excess of antibodies, Sandwich technique, directly proportional (**Figure 13**).

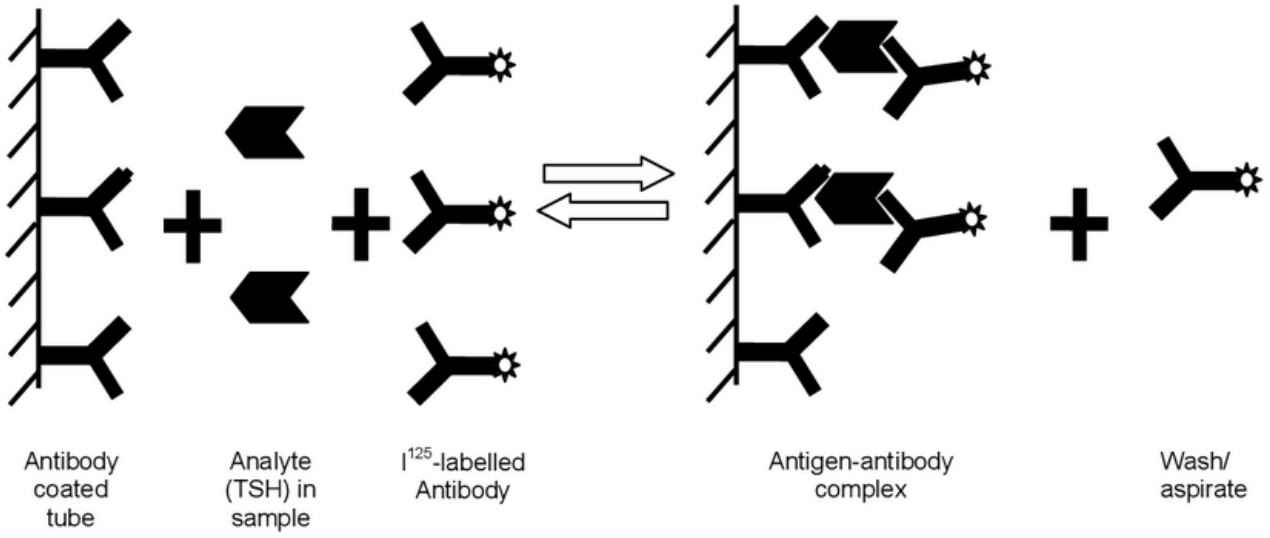


Figure 13. Immuno radio metric assay.

2.2.2. Applications

- Titration of molecules in very small quantities: hormones, traces of proteins, DNA, etc. Allergology: IgE/ Autoimmunity: anti-DNA antibodies/ Endocrinology: hormone assays/Obstetrics: beta-HCG/ Pediatrics' hematology: GH growth factors/Cancerology: tumour markers;
- Measurement of various antigens with isotypic, allotypic or idiotypic similarities;
- Immunolabelling techniques: In immunolabelling techniques, the Ag or Ac is labelled, i.e. it is coupled to an isotope (e.g. 125I), to a fluorescent compound (fluorochrome) or to an enzyme. The marker is chemically bound to the Ag or Ac, delivering a direct or indirect signal that can be quantitatively measured. The detection methods used depend directly on the type of tracer

used. Our laboratory uses special in situ detection techniques: immunofluorescence and immunohistochemistry.

- Immunofluorescence is a method that involves detecting and locating a protein of interest using a specific antibody (directly coupled to a fluorochrome or raised by a fluorescent secondary antibody);
- Immunohistochemistry by enzymatic revelation also consists of detecting and locating a protein of interest within a tissue section using a specific antibody. This antibody is revealed by an enzymatic reaction (such as peroxidase) that generates a red precipitate from an AEC (Aminoethyl carbazole) chromogen.

The table below summarises the different techniques using an enzymatic marker.

Table 1 : Enzyme immunostaining techniques

Analysis	**Dosage**	**Techniques**
Qualitative	Tissue antigen	Immunocytochemistry
	Soluble antigen	Immuno-blot and Immunodot
Quantitative	Antigenassay	CompetitionELISA
	Antibody assay	Indirect ELISA

Immunofluorescence IF

Fluorescent labelling of an antigen or antibody or an immunocomplex with a fluorescent compound (fluorochrome), a physical phenomenon characterised by the almost immediate emission of light of lower energy than that absorbed (excited molecule returns to its resting state via an intermediate state). The quantum yield thus obtained is defined by the ratio of photons emitted to photons absorbed.

2.3.1. Fluorochromes

Natural or synthetic fluorescent compounds with a visible spectrum, a high quantum yield and a fluorescence that differs from that of other biological compounds; they bind covalently to an immunological reagent; this binding must not modify the immunological properties of the conjugate (Ac, Ag or immune complex).

The usual fluorochromes are fluorescein and rhodamine.

Fluorescein: emission spectrum in the visible (# 520 nm): green colour and in the form of water-soluble fluorescein isothiocyanate (FITC),

Rhodamine: emission spectrum (# 550 nm): red in colour and in the form of lissaminerhodamine B200 (RB200) sulphonic acid chloride.

An Ac is coupled to a fluorochrome which, when excited by light (UV), will emit fluorescent light (green or red, etc.) when observed under a UV microscope. THREE types of IF:A. Direct B. indirect C. with double labelling

2.3.2. Types

2.3.2.1. Direct method (DFA)

A single fluorescent reagent with a "one-step" method. This detects the presence of an Ag, using the known specific AC, labelled with fluorescein (**Figure 14**). This substance, when subjected to an ultraviolet light source, emits light with a longer green wavelength. It is applied in:

- **Research:** immunoglobulins, autoantibodies (IgG, IgA), complement components (C3, C1q...),
- **Cell phenotyping:**Enumeration of blood populations (B, T, NK, TCD4+ and TCD8+).

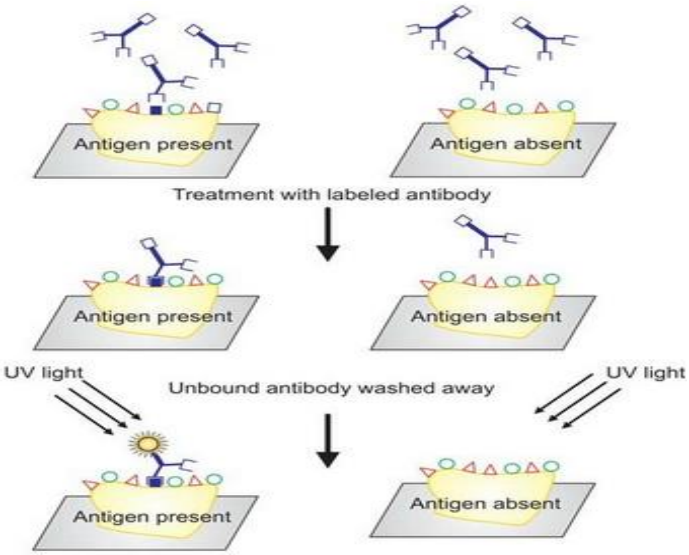


Figure 14. Direct immunofluorescence procedure.

After bringing together the reagent acting as the Ag (tissue section, cell smear) and the serum to be tested, anti-immunoglobulins labelled with a fluorescent component are added (**Figure 15**). It is the technique of choice for immunological diagnosis, screening and screening for autoimmune diseases, whether or not they are organ-specific.

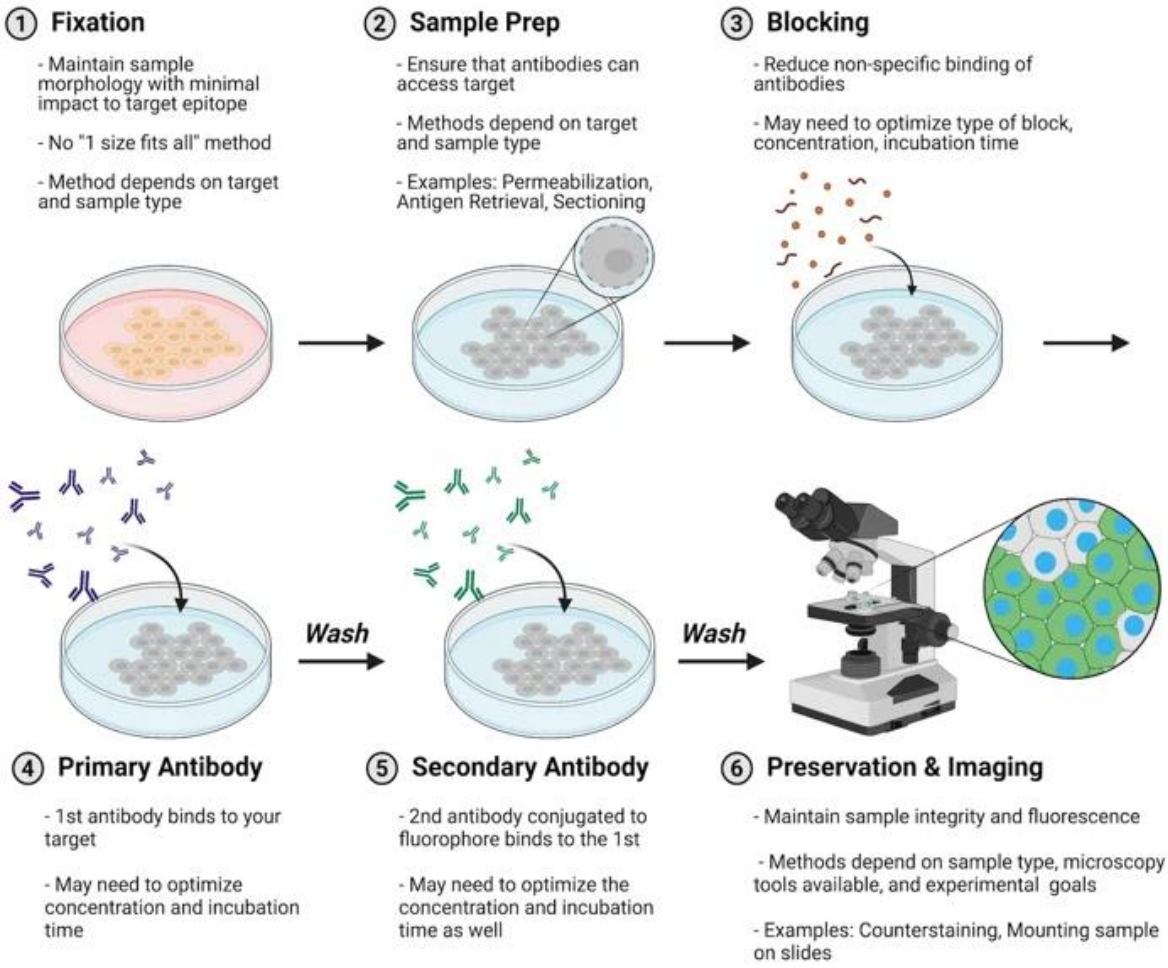


Figure 15. Indirect immunofluorescence.

2.3.2.2. Double labelling

Simultaneous detection of two antigens in a tissue section by indirect immunofluorescence technique using two markers (**Figure 16**).

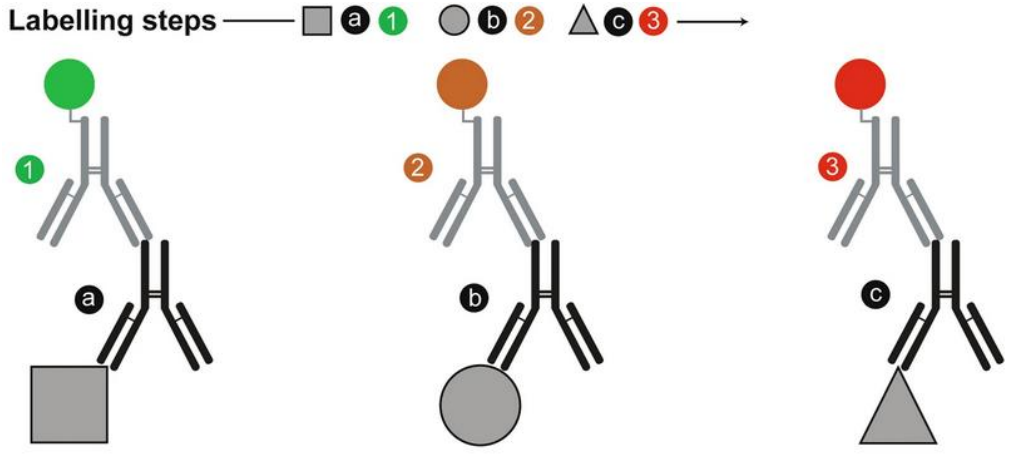


Figure 16. Double labellingimmunofluorescence.

2.3.3. Applications

Direct immunofluorescence is used to identify a germ, a class or sub-class of lymphocytes or to analyse the immunoglobulin and complement deposits in a biopsy. Several antigens or antibodies can be tested in cells, smears or tissue sections. Two reagents are used: an Ac against the first fluorescein-labelled Ag and another Ac directed against the second rhodamine-labelled Ag. The two conjugates can be added together or successively.

The fields of application are diverse:

- **Autoimmunity:**detection of antinuclear, anti-organism or organ-specific Ac,
- **Microbiology:**bacteriology, virology (kinetics of virus multiplication, detection of viruses responsible for respiratory infection),
- Basic research e.g. observation of cell division, analysis of the cell cycle including apoptosis, cell activation, molecular hybridization and intracellular localization.

Enzyme-linked immunosorbent assay: ELISA test

The Enzyme-Linked Immuno Assay (ELISA) is an immunological test used to detect or measure molecules in a biological sample. This technique is characterized by:

- Simple, practical methods that have replaced radioimmunoassay techniques;
- Colorimetric measurement of enzyme activity;
- Measurement of optical density (OD) by spectrophotometry;
- Binding of the enzyme to the antibody must not affect the catalytic activity of the enzyme or the Ag-Ac reaction.

Enzyme immunolabelling is a reaction in which the enzyme is used as a marker by association via covalent bonds with a coupling agent and the Ag or Ac (directly or indirectly). The chemical coupling agent between the enzyme on the one hand and the Ag or Ac on the other must not significantly modify the activity of either (**Table 2**).

Table 2: Methods used enzymes for attaching the enzyme to the antibody.

Enzymes	**Substrates**	**Products**
Alcaline phosphatase	PNPP (4-nitrophenyl-phosphate) coreless	PNP (4-nitrophénol) (yellow) 405 nm
Peroxydase	H_2O_2 + OPD (orthophenylen diamine)+ luminol	Coloredproduct (orange) Luminescent product
β-galactosidase	ONPG (2-nitrophényl - galactoside)	ONP (2-nitrophenol) (yellow) 405 nm
Glucose 6-phosphate dehydrogenase	Glucose 6-phosphate + NAD+ ou NADP+	NADH ou NADPH, 340 nm

2.4.1. Principle

ELISA is a detection technique that enables an antigen-antibody reaction to be visualized by means of a colored reaction produced by the action of an enzyme on a substrate previously fixed to the antibody on a solid support: microplates.

The first key stage in ELISAis the immobilization of the molecule to be adsorbed on a solid support, usually a flat-bottomed polystyrene microplate (96 or 384 wells) with a high adsorption capacity. This is a direct passive adsorption achieved by the establishment of non-covalent hydrophobic and ionic bonds between the plastic and the non-polar or ionic residues of the proteins. This step is usually carried out in an alkaline buffer such as Phosphate-Buffered Saline (PBS) pH 7.4 or carbonate-bicarbonate pH 9.6in order to ionise the molecules in solution during this step. **Figure 17** shows the general experimental protocol. A blocking step (blocking agents or detergents) is necessary as the Ac can bind to free sites in the well and give false results.

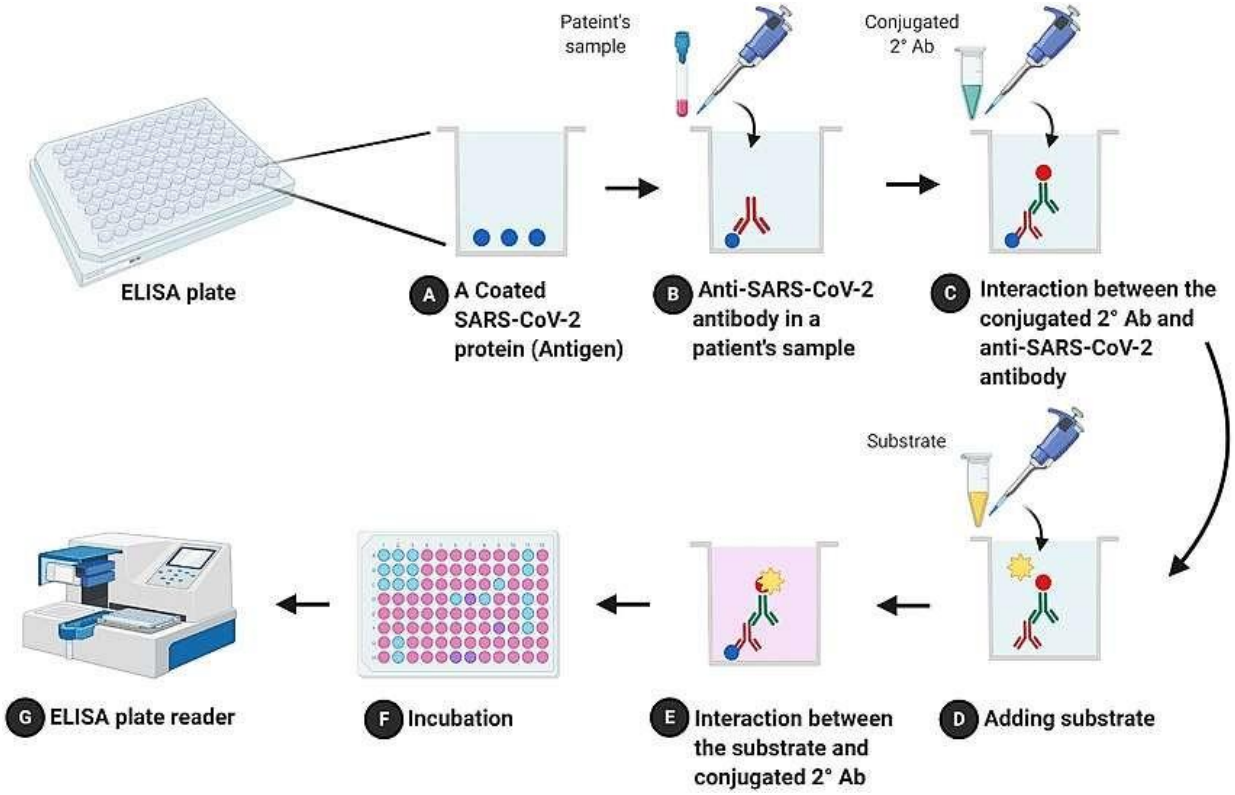


Figure17.ELISA protocol.

A SARS-CoV-2 protein (antigen) coated on the wells of the ELISA plate (A) interacts with the first antibody (anti-SARS-CoV-2 antibody) present in a patient sample (B). (C) After adding a secondary antibody (a conjugated antibody), it recognises and interacts with the first antibodies. The reaction is developed by adding a substrate (D) which is cleaved by the conjugated enzyme and changes the colour of the reaction after incubation (E) and (F), respectively. (G) The results are read by the ELISA plate reader.

The optical densityis read in an ELISA reader: the more concentrated the colour, the higher the OD, the higher the concentration of Ac or Age xp. ODs are used to obtain the concentration (e.g. of cytokine in serum). A standard curve must first be prepared with increasing concentrations of the antigen to be measured (e.g. cytokine such as IL-8 to be measured in serum) and a standard solution used (e.g. IL-8 concentrated solution). A standard curve is obtained which allows the concentration of the Ac or Ag to be measured (**Figure 18**).

	Std 1	Std 2	Des	PGE2	LPS	LPS + -5	-6	-7	-8	Neg Ctrl
6.125	0.331	0.275	0.099	0.094	0.315	0.168	0.268	0.289	0.319	0.098
3.0625	0.183	0.18	0.1	0.095		.172	0.268	0.285	0.297	0.095
1.53125	0.155	0.136	0.106	0.099	DO éch	.179	0.263	0.263	0.266	0.104
0.765625	0.139	0.13	0.105	0.105	0.322	0.205	0.278	0.298	0.279	0.102
0.382813	0.127	0.12	0.111	0.106	0.324	0.204	0.309	0.353	0.292	0.12
0.191406	0.118	0.112	0.112	0.12	0.31	0.204	0.326	0.308	0.324	0.108
	0.116	0.11	0.045	0.042	0.052	0.052	0.053	0.051	0.042	0.042
	0.123	0.123	0.044	0.052	0.051	0.052	0.054	0.052	0.052	0.053

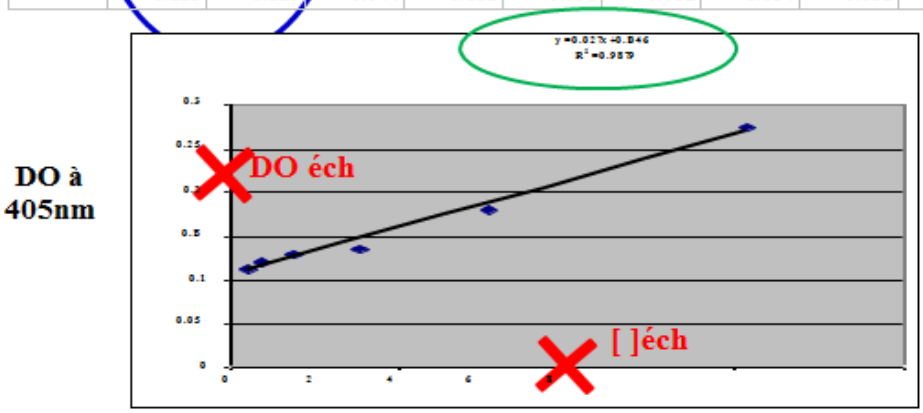


Figure 18. Standard curve for concentration measurement.

2.4.2. Types

2.4.2.1. Direct ELISA

Used to detect or measure antibodies. It uses only one primary antibody (**Figure 19**).

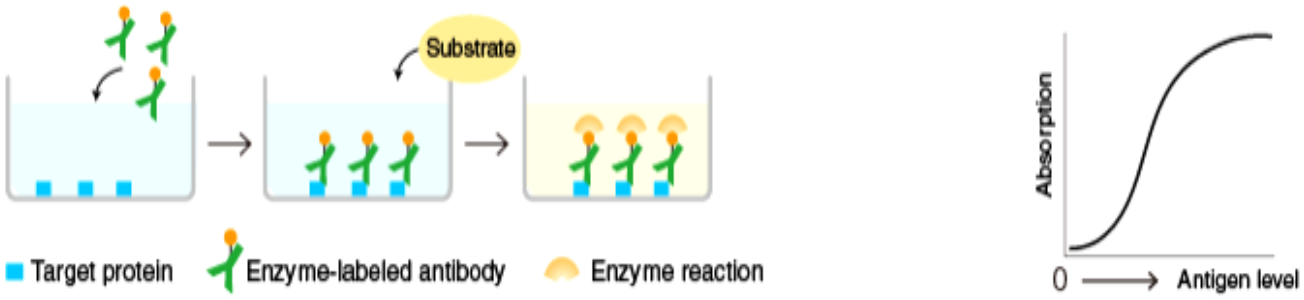


Figure 19. Direct ELISA.

2.4.2.2. Indirect ELISA

The most widely used test also detects or measures antibodies. It uses a secondary antibodywhich is more sensitive than the direct ELISA (**Figure 20**).

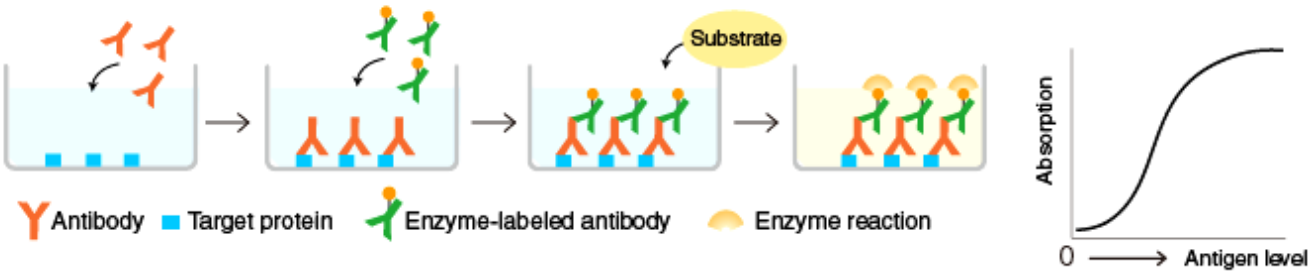


Figure 20. Indirect ELISA.

2.4.2.3. Sandwich ELISA

Two antibodies specific to two different epitopes on the target antigen are used. The capture antibody is bound to the bottom of the microplate well and binds to an epitope of the antigen. The detection antibody binds to a different epitope of the antigen and is conjugated to an enzyme that allows detection of the antigen (**Figure 21**).

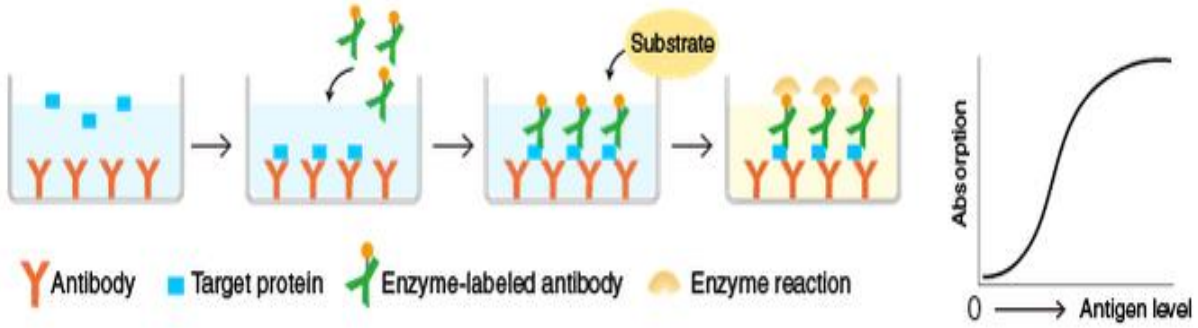


Figure 21. SandwichELISA.

2.4.2.4. Competitive ELISA

There are many assays of this type used in clinical analysis. The various stages of a competitive ELISA (**Figure 22**).

1. In the first stage: asolution of the sample, or a standard solution of the compound to be assayed, is introduced into a transparent-bottomed tube (or microplate cuvette) whose plastic wall has been sensitized, i.e. coated with the appropriate antibody attached to the surface by adsorption.
2. A known volume of a solution of the same compound is then added, covalently linked to an enzyme (this enzymatic conjugate is often peroxidase). The two forms of the compound compete during the incubation period to bind to the antibodies on the cell wall.
3. After a certain incubation time (e.g. 30 min.), the tubes are washed several times with water. Only the bound molecules remain in the tubes. The quantity of compound associated with the enzyme will be all the greater as there was little of this compound in the free state in the sample.
4. Now add the enzyme-specific substrate S and a chromogen C designed to react with the reaction product (e.g. tetramethyl benzidine).

5. The attached enzyme will transform a large number of S molecules into P species, which will react with C to give a dye. The enzyme's amplification factor makes this type of test highly sensitive. The less compound there is in the sample, the more enzyme is bound and the stronger the coloration.
6. **Finally,**the reaction is stopped after a fairly short time by adding a strong acid which destroys the enzyme. The test can then be read using a colorimeter.

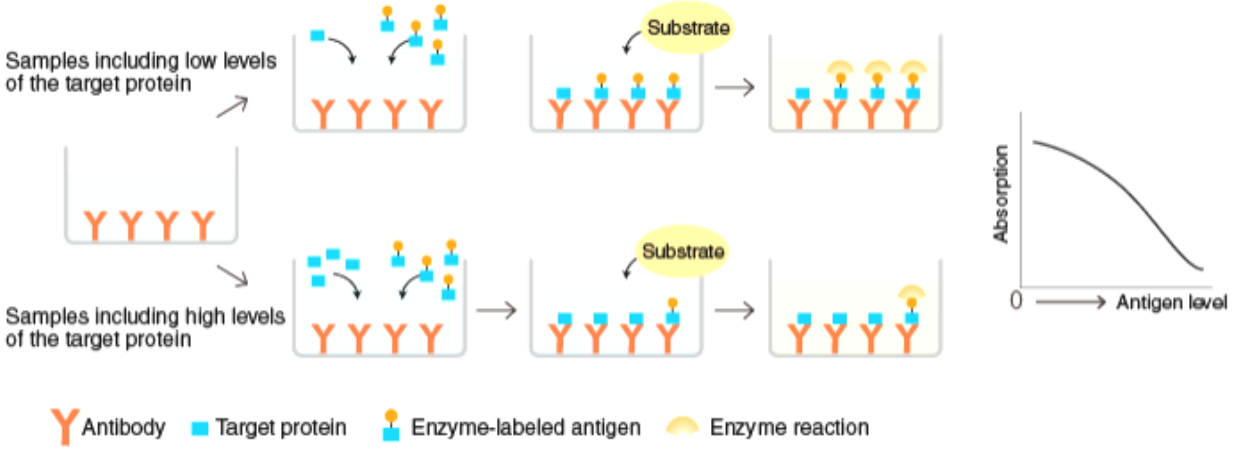


Figure 22. Competetive ELISA.

2.3.3. Applications of ELISA

ELISA can be used for quantitative or qualitative analysis:
Quantitative analysis: the optical density of the sample is interpolated on a calibration curve, usually a serial dilution of the target.
Qualitative resultsindicate the presence or absence of an antigen in the sample. Threshold values are determined by the analyst and may be based on statistics. Two or three standard deviations are generally used to distinguish positive from negative samples.
It can also be used in:
- Research and assay of AC for the diagnosis of infectious diseases (bacterial serology): in parasitology (toxoplasmosis, etc.) and virology (hepatitis B virus, AIDS virus, etc.),
- Testing for bacterial, viral, fungal and parasitic Ag, as well as autoimmune Ac, specific determination of certain plasma proteins: IgE(total and specific), ferritin, hormone assays (hCG) and drug assays, tumour marker research.

2.2.4.Advantages and disadvantages of ELISA tests

The various ELISAtechniques give accurate, reliable results. Results are obtained more quickly than by chromatography. They are primarily designed to eliminate all negative samples, so there is no need to subject them to extraction and chromatographic analysis. However, they do have a few disadvantages:

The possible recognition of several molecules with different sensitivities is a source of dispersion in the results. There is therefore a risk of cross-reaction (a false positive); the measurement range is narrow. As the concentration increases, we find ourselves in a zone of lesser precision. The correct dilution of the sample must therefore be found;

Microplates must have wells containing the same quantity of antibody with the same reactivity, which is technically difficult. Immunization of a laboratory animal is a unique event, which explains why results may differ from one kit to another. Kits must be kept cold. It is limited in time, especially for field analyses. The cost price of these tests must take into account duplicate tests and standards.

Methods for studying cell morphology: light and electron microscopy

Optical microscopy

Microscopy is a set of techniques used to obtain an image of structures at the microscopic scale. The principle is the same in all cases: a wave is directed at the sample and then emitted by the sample. This wave is captured by an objective lens, which focuses it, and passes through an eyepiece that creates an observable image. This image is either observed with the naked eye, photographed, or recorded by a camera and stored on a computer for further processing.

The purpose of an optical microscope (**OM**) is to provide access to the microscopic structure of the observed objects. A microscope is used to see finer details of the object, not just to create a magnified image. The main performance characteristic of this instrument is its resolution, meaning its ability to distinguish these fine details (**Figure 23**).

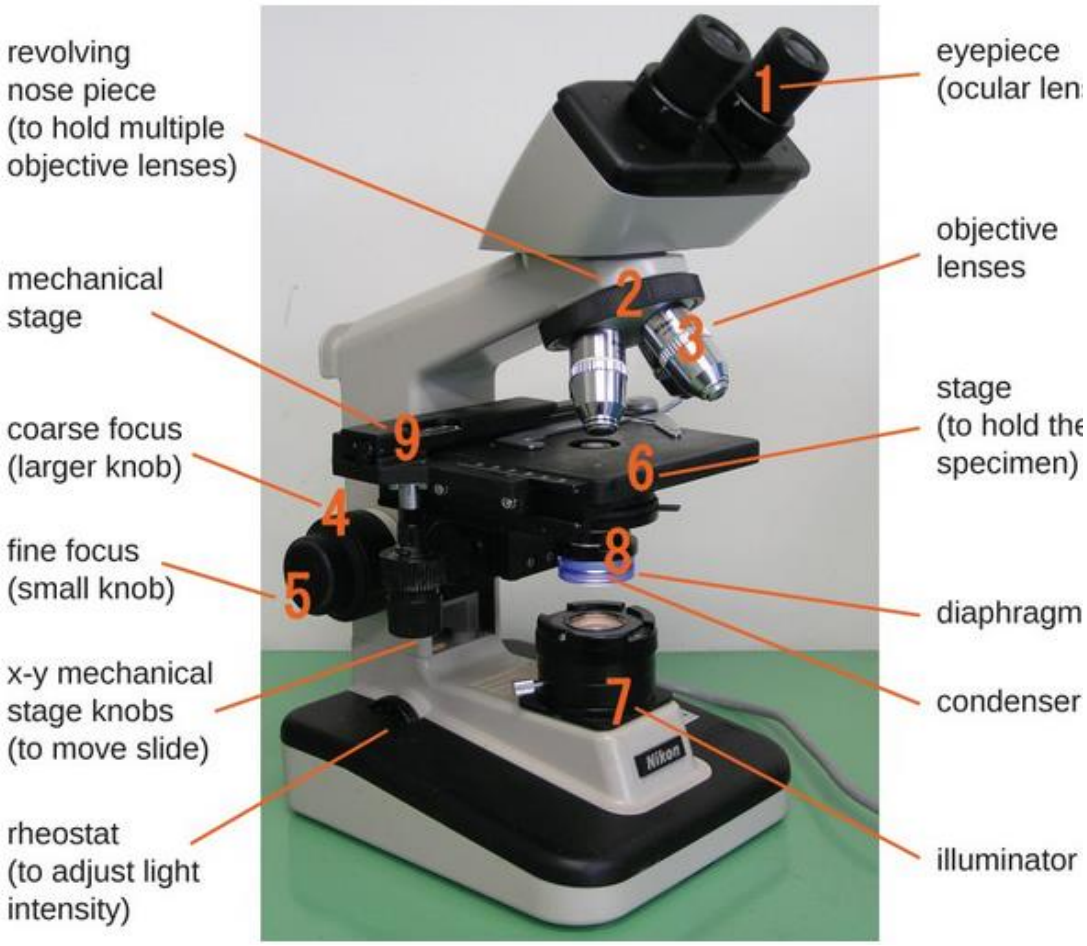


Figure 23. Components of a typical brightfield microscope.

3.1.1. Principle

The principle is as follows:the sample is illuminated by a lamp. The molecules to be observed interact with the light in several ways:

- By absorbing certain wavelengths of light. This isbrightfieldmicroscopy,
- By causing a phase shift in the different light rays. This is phase contrast microscopy,
- By emitting light at a wavelength different from the original one. This is fluorescence

microscopy.

The simplest form of an optical microscope consists of a single objective and an eyepiece, meaning two converging lenses. These two lenses form a highly magnified image of the object and allow observation of fine details in the sample. We will now go through the process of image formation step by step, using the laws of geometrical optics to trace light rays through converging lenses (**Figure 24**).

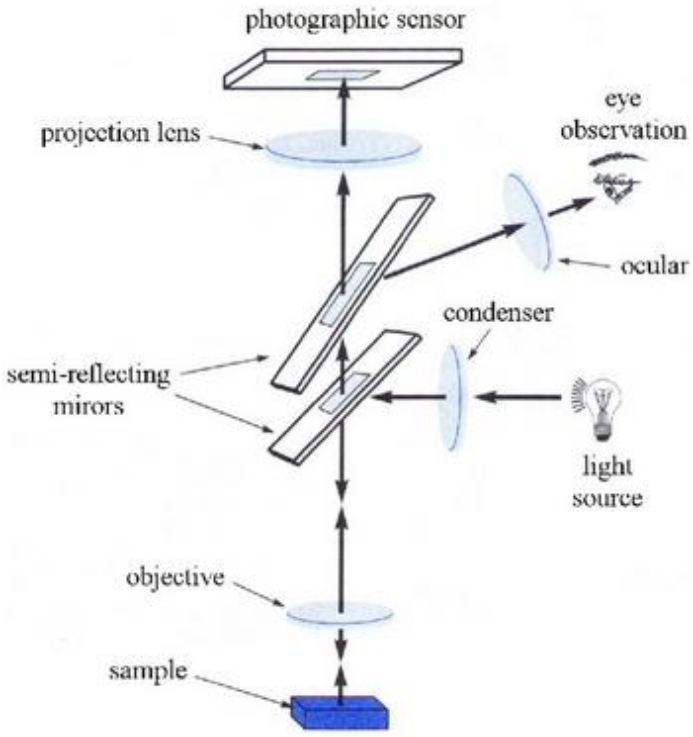


Figure 24. Schematic principle of a classical OM.

3.2.2. Types

There are several types of optical microscopes (OM), each with special optical setups designed to allow the observation of cells under certain conditions. The most commonly used microscopes are listed in **Table 3**.

Table3: Different types of optical microscopes

Types	**Used for**
Brightfield OM	Observation of internal cellular structures after staining
Darkfield OM	Observation of unstained samples and living, moving cells
Fluorescence OM	Fluorescent labeling of structures and macromolecular compounds
Phase Contrast OM	Highlighting differences in refractive indices and contrast
Inverted OM	Observation of cells in culture

3.1.3. Sample preparation

The observed sample must meet certain conditions:

- **Flatness**: The sample must be flat so that the objective can produce a sharp, entire image; otherwise, only a limited portion can be observed.
- **In Transmission**: The sample must be thin enough to allow light to pass through and make visible only a few elements (cells) in the case of biology.
- **In Reflection**: The surface must generally be polished so that scratches do not obscure what needs to be observed.
- **Differentiation of Parts**: The parts to be observed must be distinguishable. In biology, this is done through color differentiation using standard chemical staining solutions, or in metallurgy, by chemical etching with acids to reveal defects. Other differentiations can be made through polarized light, ultraviolet light (fluorescence), or interferential techniques, revealing other aspects invisible to the naked eye.

In biology,it is necessary to first place the tissue section (or liquid containing living organisms) between a slide and a coverslip (preparation of the microscopic sample between slide and coverslip, with or without staining, with or without dissection, in toto mounting, or section mounting). The objective must approach the slide for focusing without accidentally damaging the now fragile preparation.

Electron microscopy

The electron microscope (EM)was invented because the power of the optical microscope is limited by the physics of light, with a limited resolution (0.2 µm). This limit has been reached, but scientific curiosity seeks to observe the finer internal structures of cell organelles and achieve magnifications on the order of 10,000x, which was not possible using the optical microscope alone (**Figure 25**).

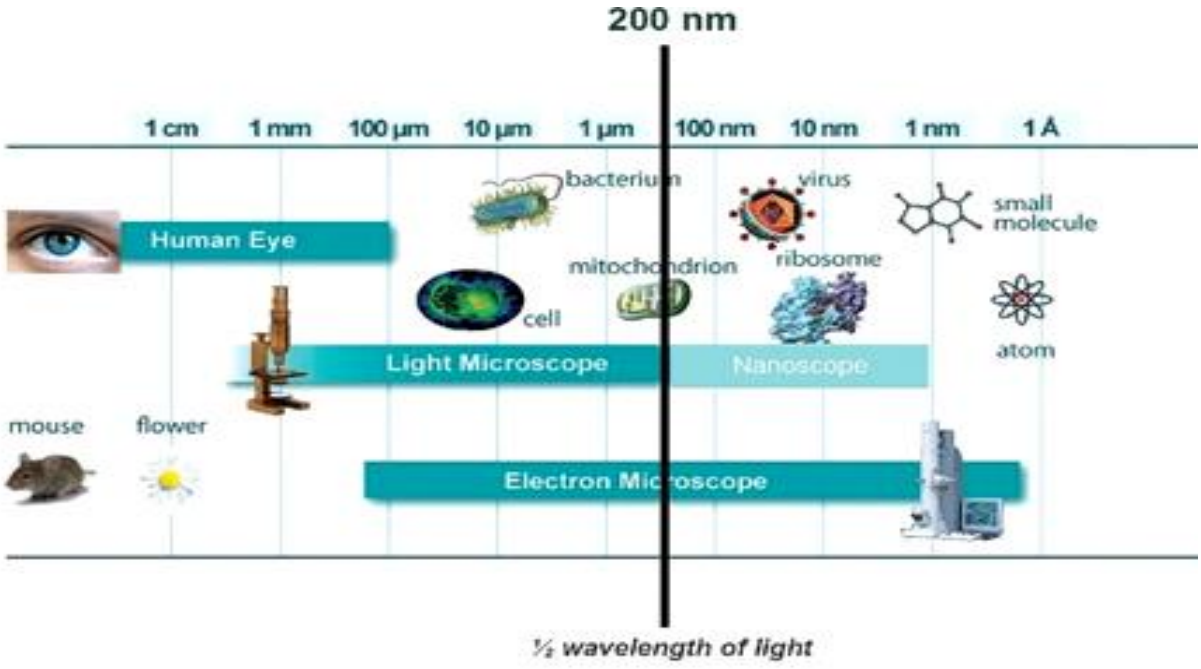


Figure 25. Microscope resolution limits.

Here is a Table outlining the main differences between a Light Microscope (MO) and an Electron Microscope (EM).

Table4: Main differences between MO and EM

Criteria	MO	EM
Source of illumination	Visible light	Electron beam
Lenses	Glass lenses	Electromagnetic lenses
Resolution	Limited by the wavelength of light (~200 nm)	Much higher resolution (up to 0.1 nm)
Magnification	Up to 1,500x	Up to 2,000,000x
Sample preparation	Simple, often requires staining	Complex, involves dehydration, embedding, etc.
Sample type	Living or non-living samples	Non-living samples only (due to vacuum)
Image type	Color or black & white	Black & white
Observation of living cells	Possible	Not possible (due to sample preparation)
Sample thickness	Relatively thick samples	Extremely thin sections required
Cost	Relatively low	Much more expensive
Operation environment	Standard air environment	Requires vacuum

3.2.1. Definition

An electron microscope is a type of microscope that uses a beam of electrons to illuminate a sample and create an image with high resolution. It operates on the same principle of lenses and beams as an optical microscope. The difference is that light is replaced by an electron beam, and glass lenses are replaced by electromagnetic lenses, which direct the electron beams for proper focusing.

Electron microscopes have a greater resolving power than optical microscopes and can achieve much higher magnifications, up to 2 million times, whereas the best optical microscopes are limited to 2000x magnification. There are two types of electron microscopes :

- **Transmission Electron Microscope (TEM)**: It allows observation of samples thin enough to be transparent to electrons.
- **Scanning Electron Microscope (SEM)**: It operates on the surface of solid objects.

The choice of electrons is determined by several criteria:

- The wavelength of the electron can be manipulated by its speed, which in turn is controlled by a positive (anode) or negative (cathode) voltage, or by the effect of an electromagnetic field applied to the electron beams.
- The low mass of these particles allows them to be easily focused using an electric or magnetic field.
- Bombardment of the sample by electron beams produces several types of emissions, but we focus on two:

Transmitted electronsthat pass through the sample: **Refracted electrons**that provide information about the sample's topography (**Figure 25**).

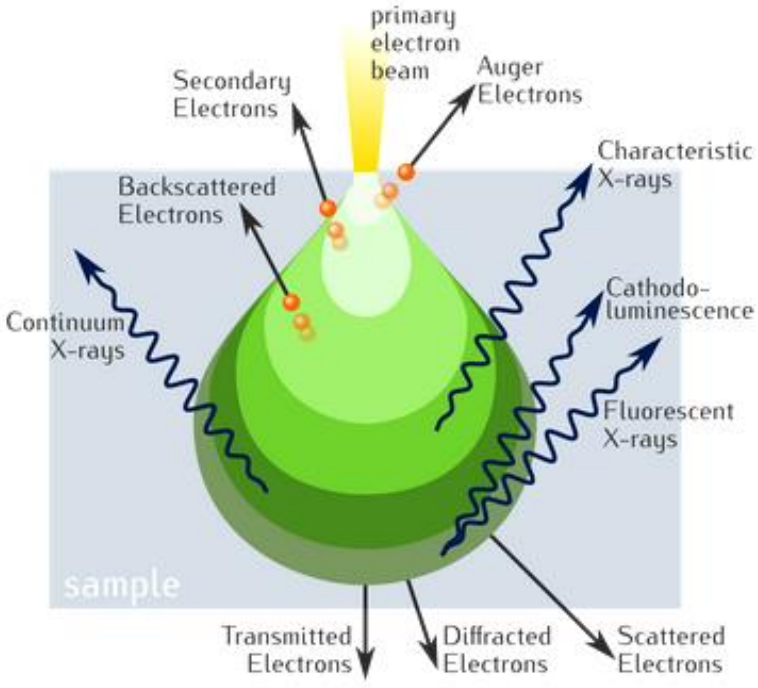


Figure 26. Various types of electronic emissions.

The main differences between the two types of ME are summarized in Table 5.

Tableau 5: Comparaison between SEM etTEM

Criteria	**SEM**	**TEM**
Type of image	Surface	Cellular component
Sample placement	After objective lens	Before objective lens
Electron involved	Refracted	Transmitted
Type of sample	Mineral or biological	Often cellular
Preparation	Metallization	Embedding
Detector	Present	Absent
Screen	Fluorescent	Electronic
Scanning	/	Absent
Projectorlens	/	Present

3.2.2. Components of EM

The main components of an electron microscope are presented in **Figure 27**.

Figure 27. Major components of an electron microscope.

3.2.2.1. Electronic control

This is the only link between the user and the instrument's electronics. All measurements are digital, and all currents and voltages are set by power amplifiers to control magnification and image contrast. It also allows for the recording of these images.

3.2.2.2. Vacuum pump

There are numerous reasons why a good vacuum must be maintained inside the column:

- To prevent oxidation of the sample or the tungsten filament.
- To prevent contact between electrons and air particles.

3.2.2.3.Liquid Nitrogen Pump

Used for cooling the column due to the heat emission caused by the use of high voltage.

3.2.2.4. Column

The column contains the sample and all the components (electron gun, lens, and screen) necessary to produce the image:

- **Electron Source**:The electron gun consists of a tungsten filament that is heated by passing an electric current. Electrons are extracted from the filament and form a cloud around it. The emitted electrons are then accelerated down the column towards an anode by the high voltage. However, the diameter of the electron beam is adjusted by the Wehnelt electrode (which is at a negative potential) to focus the electrons into a well-defined beam (**Figure 28**).

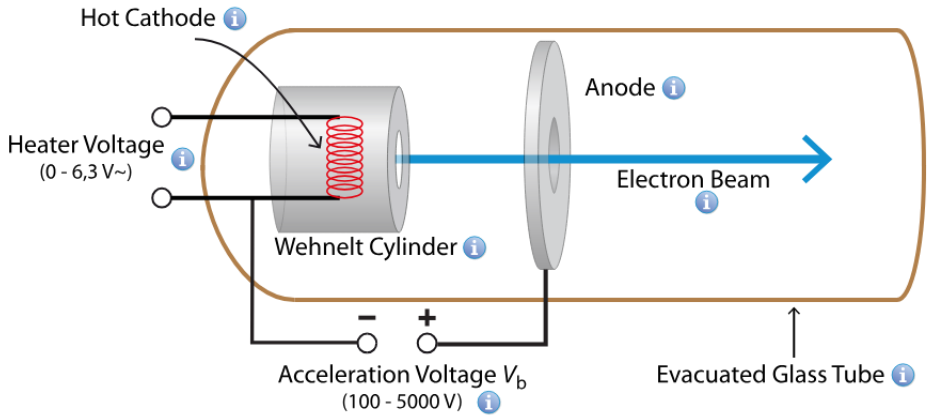


Figure 28. Electron canon.

The lenses are responsible for accelerating the electrons to control the wavelength and adjust the amount of surface area to be illuminated. A magnetic lens is a device that produces a symmetric magnetic field to focus the electron beam (**Figure 29**). The screen is coated with a thin layer of phosphorescent elements, called luminophores, which emit light when excited by electrons striking the surface, forming bright spots

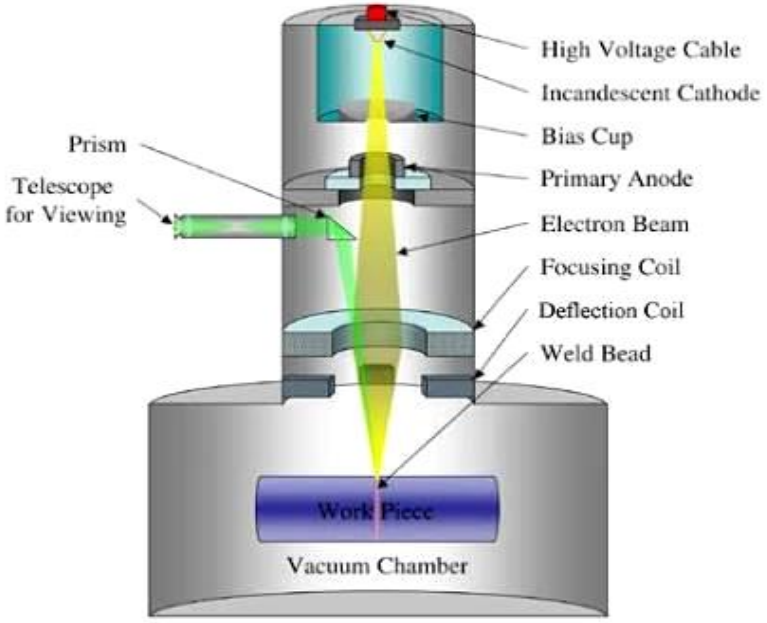


Figure 29. **S**chematic diagram of electron beam machining process.

3.2.3. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is an electron microscopy technique capable of producing three-dimensional images of the surface (topography) of a sample with very high resolution, on the order of 0.8 nm, and a magnification of up to 200,000x, using the principle of refracted electrons. The image is progressively formed by scanning the sample surface with a well-focused electron beam.

3.2.3.1. Sample preparation

Biological samples contain a high proportion of water, and damage can occur when introducing these samples into the vacuum environment of the column. The sample preparation steps are as follows:

1. **Fixation**: This step aims to prevent abrupt deformations of the protein or lipid substances in the sample, which occur when the sample is placed under vacuum in the column. Fixation is typically carried out chemically, using glutaraldehyde, followed by osmium tetroxide.
2. **Dehydration**: This process involves replacing the water in the cells with a hydrophobic liquid such as alcohol or acetone, by passing the sample through successively higher concentrations of alcohol baths.
3. **Metallization**: The sample is coated with a thin layer of metal. This involves heating a metal filament under vacuum to evaporate it. The metal atoms will then deposit on the sample to form a thin coating.

3.2.4. Operating principle

- The SEM column consists of an electron gun that produces an electron beam,
- Condenser lenses are used to adjust the diameter and intensity of the electron beam,
- The objective lens focuses the electron beam onto the surface of the sample to achieve focusand the desired magnification,
- The deflection coil (scanning coil), located inside the objective lens, moves the focused beam across the sample in two perpendicular directions to scan the entire surface,
- A sample stage inside the chamber holds the sample in place and allows for tilting and rotation,
- The interaction between the electron beam and the sample causes the emission of "secondary electrons," which are collected by an electron detector,
- To study the topography of a sample, the detector measures the intensity of secondary electron emission and converts it into a bright point on the screen. The quantity of secondary electrons produced depends not on the chemical nature of the sample, but on the angle between the incident beam and the surface (**Figure 30**).

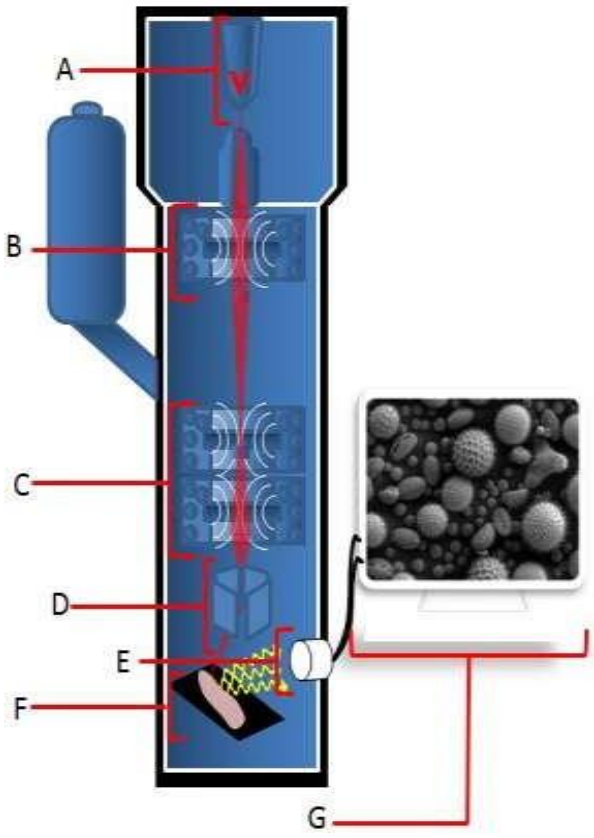


Figure 30. Principle of SEM operation.

3.2.5. Transmission Electron Microscopy (TEM)

A transmission electron microscope is capable of producing detailed images of the morphology of samples with a resolution of 0.08 nm and a magnification of up to 200,000x. It is used to study the morphology, ultrastructure, and localization of cellular organelles, and is commonly used in the fields of cytology and microbiology. The principle involves collecting electrons transmitted through ultrathin sample sections, typically 60 to 90 nmthick.

3.2.5.1. Sample preparation

1. **Fixation**: This step preserves the structures of the sample by immersing it in glutaraldehyde and osmium tetroxide.
2. **Embedding**: The water in the tissues is replaced by resin. The embedding process is carried out by passing the sample through resin-solvent baths of increasing concentration. The resin is then solidified by heat polymerization, creating a hard block that is stable under the electron beam, allowing for the preparation of ultrathin sections.
3. **Preparation of ultrathin sections**: For TEM observation, ultrathin sections of the sample (resin block) are required. These sections are produced using an ultramicrotome, which cuts sections 10 to 100 nm thick, increasing the amount of transmitted electrons.
4. **Enhancing contrast of the sections**: To improve the contrast between different cellular organelles, the sample sections are exposed to a solution containing heavy atoms (uranyl acetate and lead citrate). These atoms bind to the membranes of cellular organelles, such as the nucleus, Golgi apparatus, and mitochondria, making them more visible.

All of these steps are illustrated in **Figure 31**.

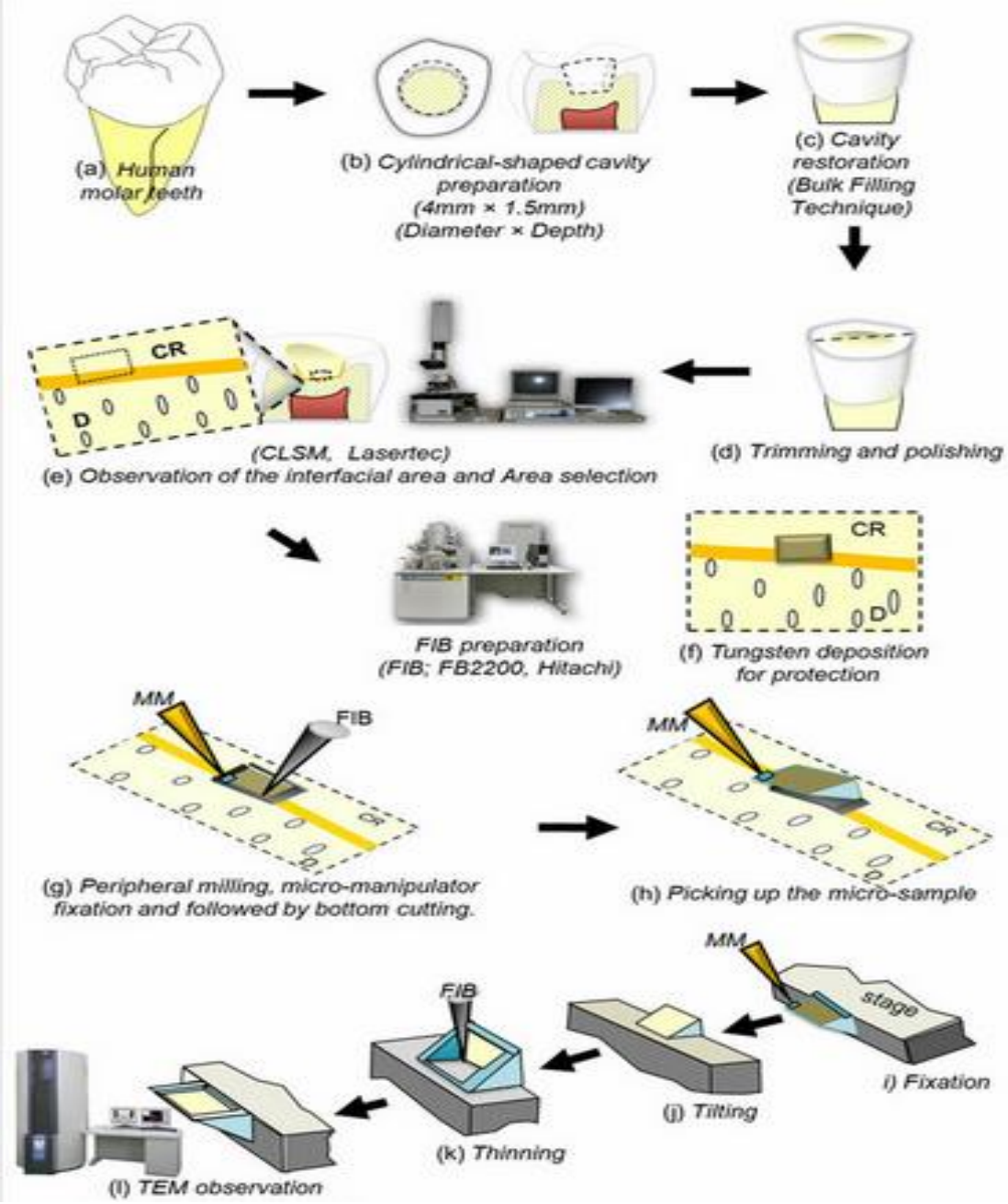


Figure 31. Schematic diagram showing the steps of specimen preparation for TEM examination.

3.5.2.2. Principle and operation

A. The scanning Electron Microscope (SEM) column consists of an electron gun that produces an electron beam.

B. The condenser lenses are used to adjust the diameter and intensity of the electron beam to illuminate the sample, which is placed just after the condenser lens.

C. Below the sample is the objective lens, which collects the transmitted electrons.

D. The projector lens then projects the beam from the objective lens onto a fluorescent screen, which forms an image.

E. Electrons passing through the sample can be deflected, with the likelihood of deflection increasing with the charge of the sample's constituents. The more charged areas therefore appear darker, while areas with little or no charge appear lighter in the image.
F. The electron beam is converted into luminous spots on a fluorescent screen (**Figure 32).**

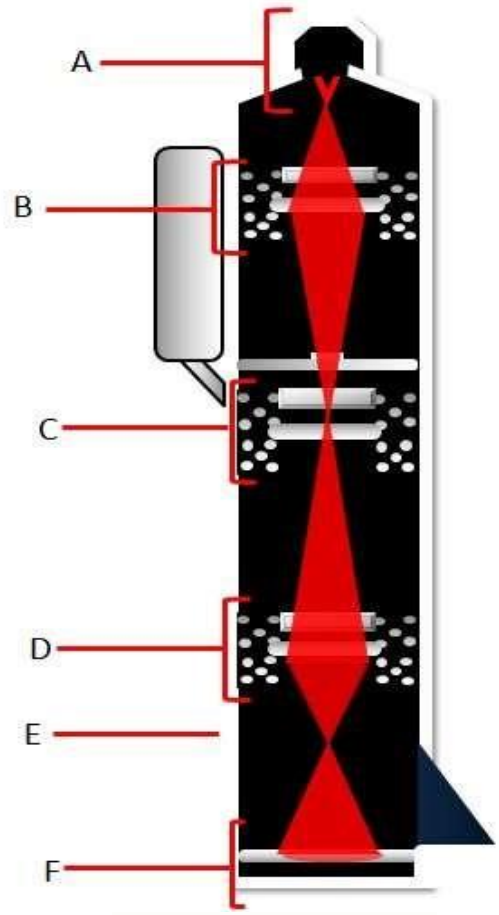


Figure 32. Operating principle of a TEM.

3.2.6. Fluorescence microscopy

In general, biological molecules (proteins, acids) are not fluorescent in the visible spectrum (with a few exceptions: flavins, NADH, fluorescent proteins, etc.). However, some amino acids do exhibit fluorescence in the UV spectrum (e.g., tryptophan). Specific fluorescent markers are attachedusingmethodssuchas:

- Covalent coupling (chemicalbonding);
- Affinity labeling: a fluorescent molecule is used that binds specifically (e.g., antibodies, toxins, etc.);
- Cloning a fluorescent protein.

Fluorescence microscopy provides a completely different type of contrast compared to previous techniques. Instead of highlighting changes in the refractive index of the sample with transmitted light,

it involves marking molecules or structures of interest with a fluorescent probe that emits its own light. This way, only what has been specifically marked is observed, while the rest of the sample remains dark, providing a much stronger contrast than previous methods. This allows for the detection of single molecules and monitoring their dynamics (**Figure 33**). Known types of fluorescence microscopyinclude:

- Epifluorescencemicroscopy,
- Confocalmicroscopy,
- Evanescent wavemicroscopy,
- Two-photon microscopy.

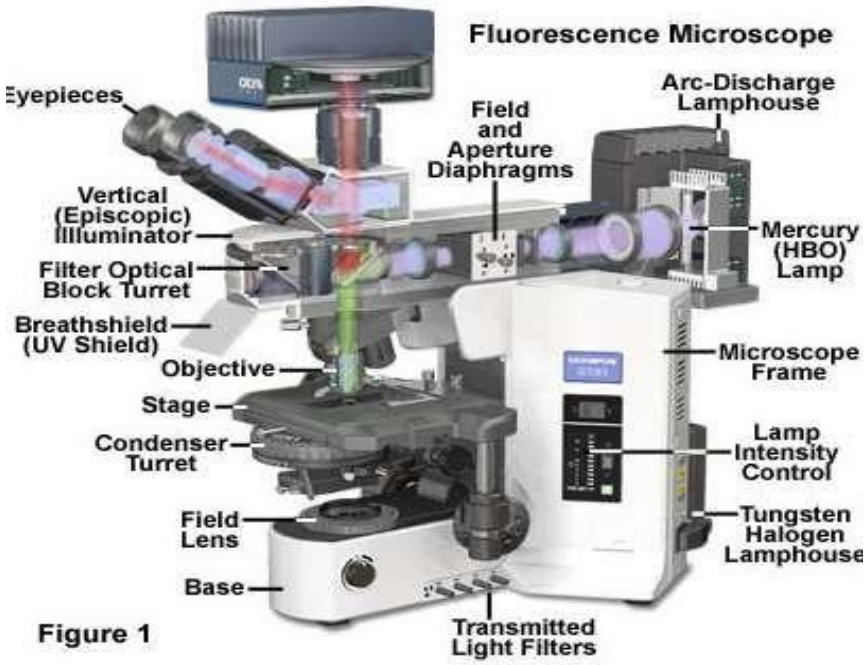


Figure 33. Fluorescence microscope.

3.2.6.1. Principle

The fluorescence microscopehas the same components as a transmission microscope (generally, both types of illumination are available on the same microscope), except for the light source, which has special properties, and the presence of filters that allow spectral selection. In the most common setup, the microscope also uses epifluorescence illumination, meaning the exciting light passes through the objective lens, which then serves as the condenser (**Figure 34**). This implies that the numerical aperture of the condenser is the same as that of the objective without any adjustments, and the resolution remains optimal.

The most commonly used light source for fluorescence is the mercury vapor lamp. It provides powerful and stable illumination, and the emitted light spectrum has several peaks that can be used to excite common fluorophores.

Filters are used to select the range of wavelengths that will be used to excite the fluorophores or to collect for imaging.

Beyond wavelength selection achievable with a filter, it is sometimes necessary to extract more information from the fluorescence emitted by the observed sample. To achieve this, the emitted light can be dispersed according to its spectral properties in various ways. Two examples of this are presented here: prisms and diffraction gratings.

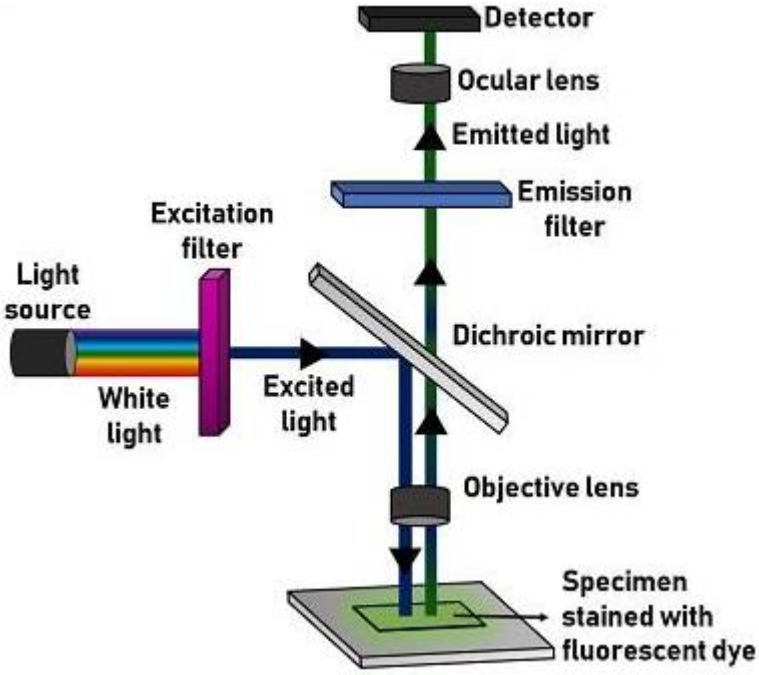


Figure 34. Working principle of fluorescence microscopy.

3.2.6.2. Applications

Fluorescence microscopy is widely used in biology, particularly in cell biology. One of the most common applications is the labeling of a protein by immunofluorescence, which involves using a primary antibody against the protein of interest, followed by a secondary antibody directed against the primary one, which is coupled with a fluorophore. This method allows for the study of the localization of endogenous proteins within the cell and potential translocations following treatments, for example (**Figure 35**).

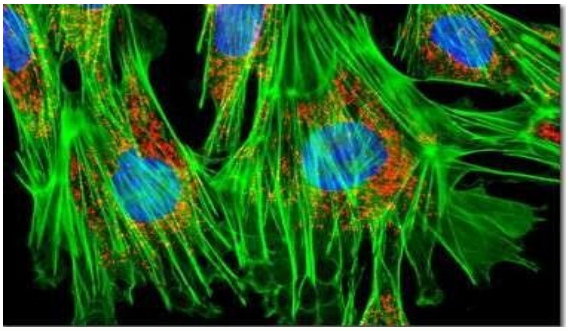


Figure 35. 3T3 cells labelled with MitoTracker (mitochondria, red), phalloidin-Alexa488 (actin, green) and DAPI (nuclei, blue)

In addition to the direct applications of fluorescence, there are many derived techniques such as FRAP, FRET, FCS, and others. These techniques provide additional information beyond simple

molecular localization, such as their mobility (diffusion, speed) or their interactions with other molecules.

The use of fluorescence, especially for quantitative or even semi-quantitative studies, requires caution and awareness of potential artifacts. The two most frequently encountered issues are photo-bleaching (loss of signal during illumination due to the photo-destruction of fluorophores) and image saturation (which masks fluorescence variations and thus leads to significant loss of information).

Flow cytometry and tricellular analysis

Flow cytometry

4.1.1. Definition

flow cytometry describes a technique for the routine analysis of cells or biological particles in suspension that pass through a measuring cell one after the other. One or more lasers excite each injected particle. If the particles have been labelled with one or more fluorochromes, the light source excites them and provides information on certain additional biological aspects.

The cytometer measures fluorescence and scattered light. Using light scattering phenomena, it characterises cells according to:

- Size (in the laser axis),
- Granularity (at 90° to the laser),
- fluorescence (**Figure 36**).

Figure 36. Flow cytofluorimeter.

4.1.2. Principal techniques

To operate, a flow cytometer requires a combination of:

- A fluidic system to introduce and channel the cells and bring them to the laser;
- An optical fluid system consisting of the laser(s) and a system of filters to excite, recover and amplify the various signals emitted;
- Electronic fluidics system to convert the optical signals into proportional electronic signals and digitize them;
- A fluidic computer system to display these signals.

Cell samples must be suspended before they can be analyzed. Blood analysis poses no problem, as the cells are already in suspension. However, the cellular tissues must be dissociated and the aggregates eliminated so that they can be analyzed.

- Fluidic system

The cellsare brought to the centre of the measuring nozzle or a narrow laminar flow and aligned one behind the other (using the hydrodynamic sample centring system) in order to be excited by one or more light beams: this is hydrodynamic centring. It uses an air pressure regulator to ensure stable

operation, supply of sheath liquid and injection of the sample into the measuring cell. This assembly enables the particles to be targeted at the point of impact of the laser.

After passing through a succession of mirrors and filters, the light is collected by the detectors and converted into an electrical signal by a PMT photomultiplier or PD photodiode. The optical signals collected have an intensity correlated with cellular properties (**Figure 37**).

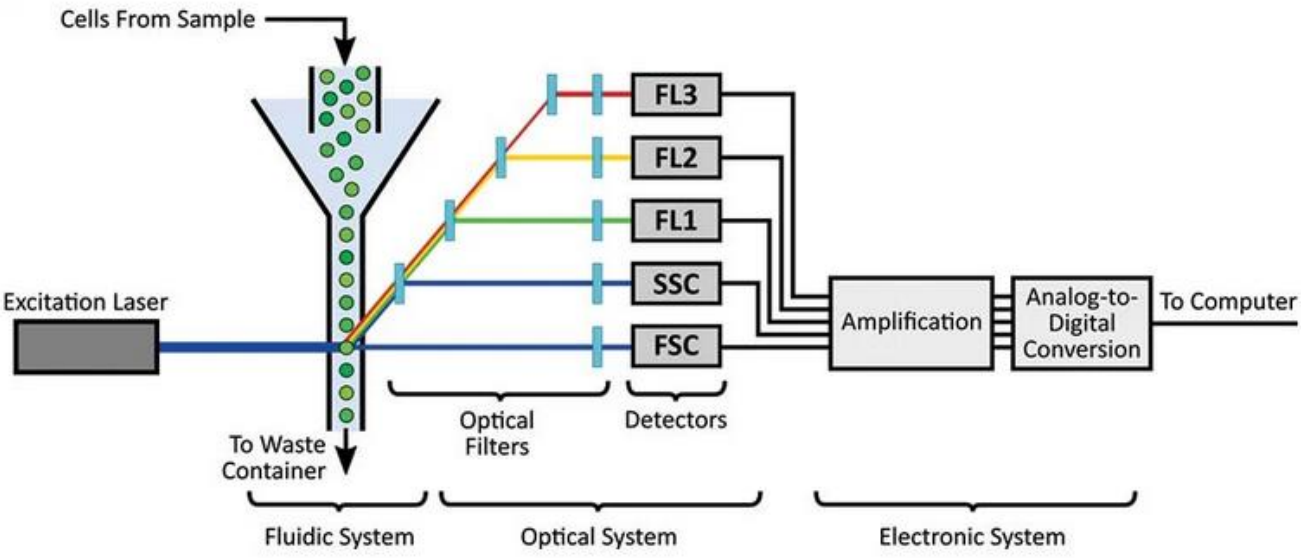


Figure 37. Schematic of a common flow cytometer, illustrating the fluidic, optical, and electronic systems.

4.1.2.1. Optical system

A light source needs to be focused on the cells to illuminate the dyes at a wavelength close to their excitation maximum (**Figure 38**). Two types of source are currently used:

- **Lasers.**Each emits monochromatic light capable of exciting a series of fluorochromes. Lasers have discontinuous emission spectra. There are a number of different lasers: for example, argon ion lasers can excite fluorescein, phycoerythrin and propidium iodide at 488 nm and in UV light;
- **Mercury vapour or xenon lamps**are also used. They are less focused than lasers, but the spectrum is fairly road and their cost is limited.

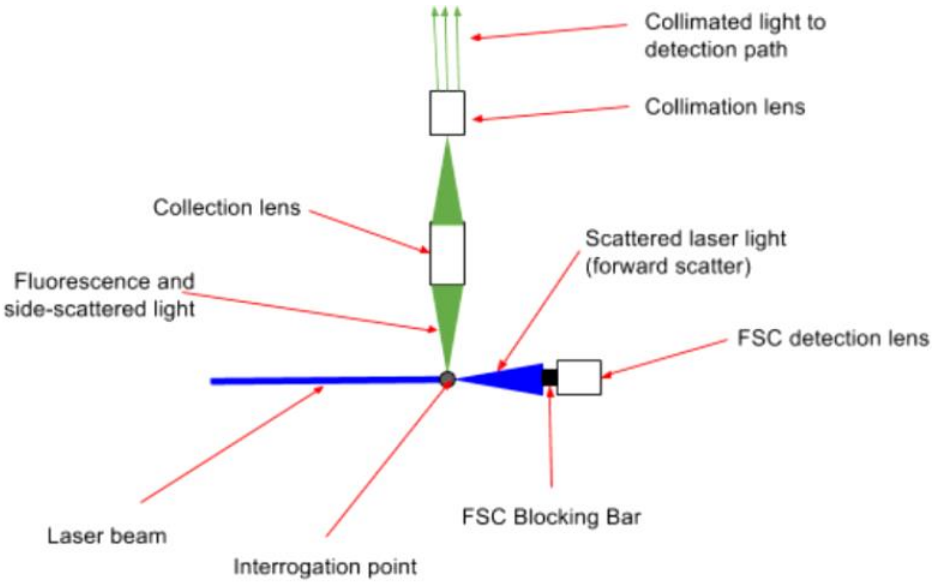


Figure 38. Example of a flow cytometer light path.

4.1.2.2. Electronic system

Optical signals are converted into electrical signals by photomultipliers (PMTs). PMTs are optical sensors capable of detecting very small amounts of light and converting it back into an electrical signal, either digital or analogue depending on the cytometer. They will therefore translate the intensity of the light reaching them(**Figure 39**).

Figure 39. Electronic card.

4.1.2.3. Computer system

The numerical values obtained from the converters are stored by the computer in various media and displayed on the cytometer screens as histograms (for one parameter), where the x-axis represents

the intensity of the analyzed signal and the y-axis represents the number of cells, or as cytograms (for two parameters).

4.1.2.4. Collected signals

Flow Cytometry (FCM)is a technology that enables the quantitative, non-destructive, and rapid analysis of isolated cells, analyzed individually at a rate of 100,000 cells per minute. Indeed, the intensity of the optical signals collected correlates with certain cellular properties (**Table 5**):

- **Scattered light**provides information on the morphology and structure of the cell. If the light scattering is measured along the axis of the incident beam, the signal intensity can be correlated with the cell's size and viability. At a 90° angle, the measurement corresponds to the cell's intracellular structure (cytoplasmic refringence, morphology, nucleus-to-cytoplasm ratio). Using both parameters simultaneously allows the distinction between platelets, lymphocytes, monocytes, and polymorphonuclear cells in peripheral blood, for example.
- **Absorbed light**increases proportionally to the cell diameter (assumed to be spherical) and the absorption index of the cellular constituents.
- **Emitted fluorescence**may be spontaneous, but most often, it is introduced into the cell via a fluorochrome.

Table 5: Significance of the main signals obtained in FCM

Parameters	Significance	Uses
Light scattering at small angles	Proportional to the cell diameter	Morphological identification of cells
Light scattering at right angles	Proportional to cellular content	Morphological identification of cells
Fluorescence	Proportional to labelingintensity	Cellular markers, DNA, RNA, cell functions

4.1.3. Operating principle

Flow Cytometry involves analyzing optical or physical signals emitted by a particle intersecting a laser or lamp light beam. The signals are separated by optical filters, collected by photomultipliers, amplified, digitized, processed, and stored by a computer. This "cell-by-cell" analysis is multiparametric and can be performed at a rate of several thousand events per second (**Figure 40**).

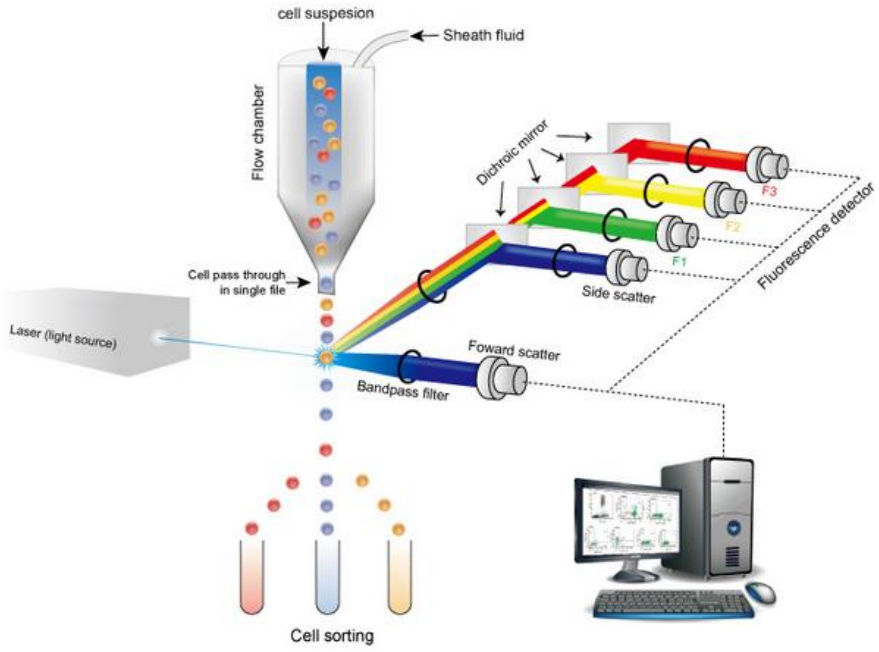


Figure 40. General diagram of flow cytometry analysis.

4.1.4. Presentation of results

The numerical values obtained from the converters are stored by the computer and displayed on the cytometer screens in two forms:

- **Monoparametric histograms**, where the x-axis represents the intensity of the analyzed signal, and the y-axis represents the number of cells.
- **Biparametric histograms or cytograms**, showing two signals simultaneously.

4.1.5. Types of representation

Several types of representation can be mentioned:

- **Graphical representation**shows relative fluorescence in relation to the number of events.
- **Dot plot (Size and granularity representation)**: For each cell that passes through, its size is analyzed in relation to its granularity. This is an effective way to detect a small number of events where populations are clearly separated.
- **Density plot (Relative density of events representation)**: This representation simulates a 3D plot, where the third parameter is the number of events. This allows the user to highlight a discrete population.
- **Three-dimensional histogram**: Obtained from a biparametric plot with a third dimension added, representing the number of cells. This helps visualize the proportion of different cell categories in relation to one another (**Figure 41**).

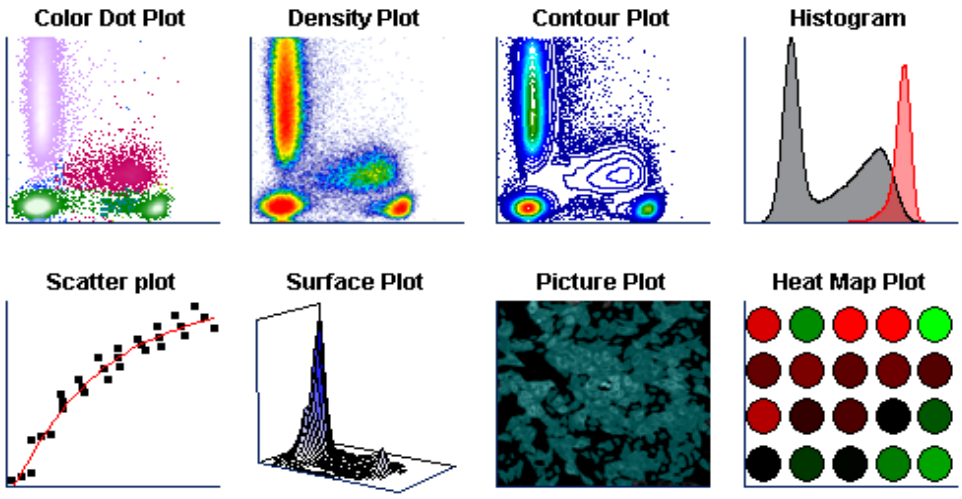


Figure 41. Examples of different ways of visualizing flow cytometry data.

4.1.6. Software

There are several CMF acquisition and analysis software packages. Examples include Cyview, Cellquest and Flomax. Software is used for acquisition (compensation settings, etc.), analysis (population percentages and comparison of results) and formatting of the information obtained (date, time, sample information). They can be used to display 'windows', each with its own shape (**Figure 41**).

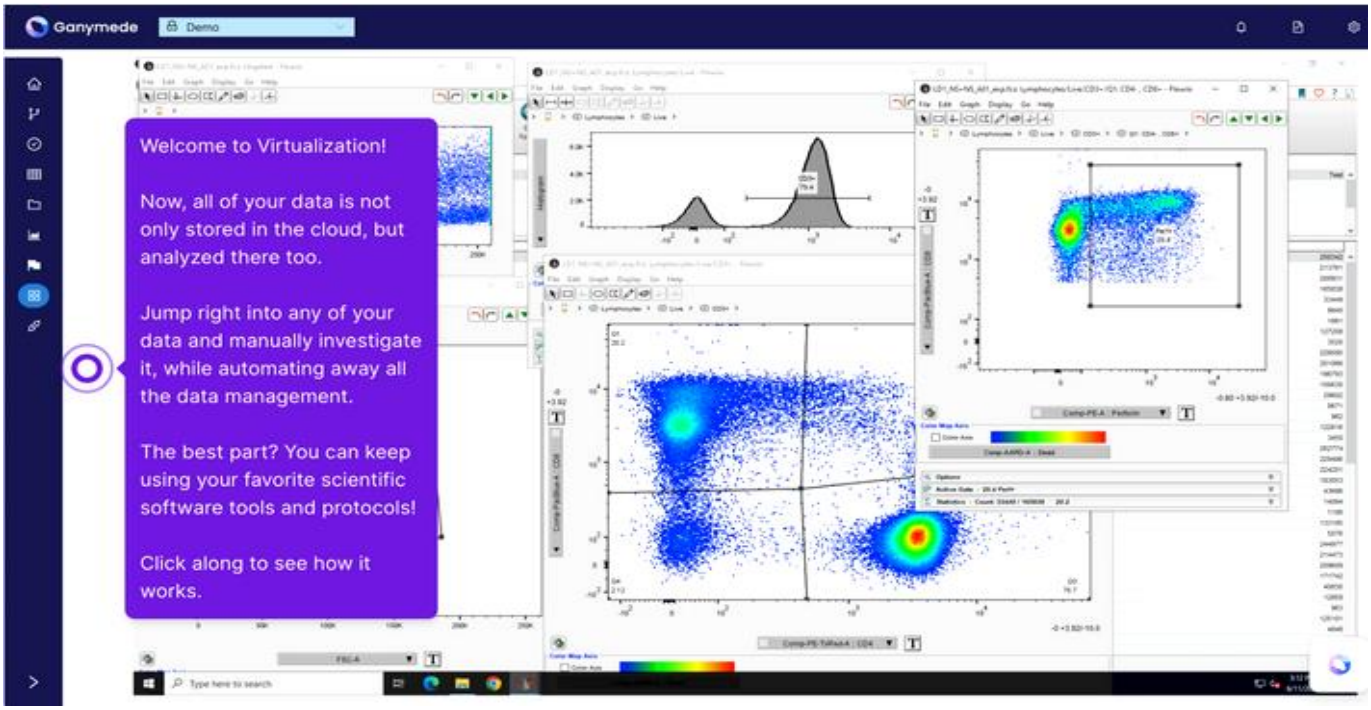


Figure 41. Software example flow cytometry.

4.1.7. Applications

Several applications of flow cytometry are used in routine practice, including:

- **Immunophenotyping**(hematology, immunology): Some of these applications are now regularly used for diagnosing or monitoring the treatment of various conditions, particularly malignant hematologic diseases.
- **Quantitative expression of surface or intracellularantigens**.
- **DNA quantification**(oncology, cytogenetics): The detection of pathological cells is the most developed application. This detection is primarily based on measuring abnormal DNA content in the nucleus of tumor cells.
- **Study of the cell cycle**: FCM offers a quick and simple methodology for analyzing the cell cycle. It allows the tracking of the distribution of cells in the different phases of the cycle based on various stimuli or the addition of certain drugs. It also enables the detection of cells with abnormal DNA content.
- **Plant physiology**(ploidy, DNA content, for selecting the most resistant plants).

Additionally, fundamental research applications include:

- Flow karyotyping.
- Calcium flux.
- Intracellular pH.
- Membrane fluidity.
- Measurement of oxidative stress.

4.1.8. Advantages and disadvantages of FCM

4.1.8.1. Advantages

What sets FCM apart from other analytical and preparative techniques is that it combines the following five essential features: quantitative analysis, detection sensitivity, speed, multiparametric cell-by-cell analysis, and sorting.

- **Quantitative analysis**: With a microscope, it is difficult to classify cells into more than four categories based on their fluorescence: "negative," "low," "medium," and "high." A flow cytometer with a logarithmic amplifier allows for precise quantification of each optical criterion over a range of 1 to 10,000 arbitrary units of fluorescence. However, given the number of parameters involved in any FCM analysis (optical settings, fluorochromes, markers), absolute quantification requires the use of calibrated standards (e.g., fluorescent beads).
- **Detection sensitivity**: In immunofluorescence, it is possible to distinguish from background noise a population of lymphoid cells carrying about 1,000 antigenic determinants per cell.
- **Acquisition speed**: The average analysis speed of a cytometer is 1,000 cells per second, although modern devices can reliably analyze up to 10,000 events per second across several parameters.
- **Simultaneous analysis of multiple parameters**: FCM allows for the simultaneous analysis of multiple parameters, making it possible, for example, to measure two or three parameters at the same time on a population of lymphocytes from blood or bone marrow. No other method, whether physico-chemical or immunological, offers such versatility.

- **Sorting**: Cells can be isolated with purity rates exceeding 99%. These cells can be recultivated. However, achieving such high purity of sorted populations through FCM comes at the cost of a significant reduction in the number of collected cells and requires constant monitoring during separation.

4.1.8.2. Disadvantages

- The cells must be in suspension.
- The number of cells must be at least a few hundred thousand.
- No images of the analyzed cells are available.
- Since the analysis occurs at a single point in time, it is impossible to conduct a true kinetic study of the same cell.

Cell Sorting

Based on the FCM (Flow Cytometry) technique, the cell sorter allows the isolation and retrieval of one or more cell populations according to criteria such as size, granularity (cellular content), and/or fluorescence (receptor, genetic transformation, cell cycle phase, etc.). Cell sorting can range from enriching a population to selecting a fully pure type of cell. The collected cells can then be analyzed biochemically or recultivated.

4.2.1. Definition

Cell sorting is one of the earliest applications of FCM. Essentially, cells are separated according to the information provided by their phenotype (**Figure 42**).

Cell sorting is a method for physically isolating targeted cells based on their morphological and fluorescent characteristics within a heterogeneous mixture. Cell sorting enables a wide range of downstream applications, such as cell culture, molecular and cellular analysis, or cloning individual cells. Our instruments have the capacity to collect cells in bulk (up to 4 populations simultaneously) or as individual cells (plates up to 384 wells).

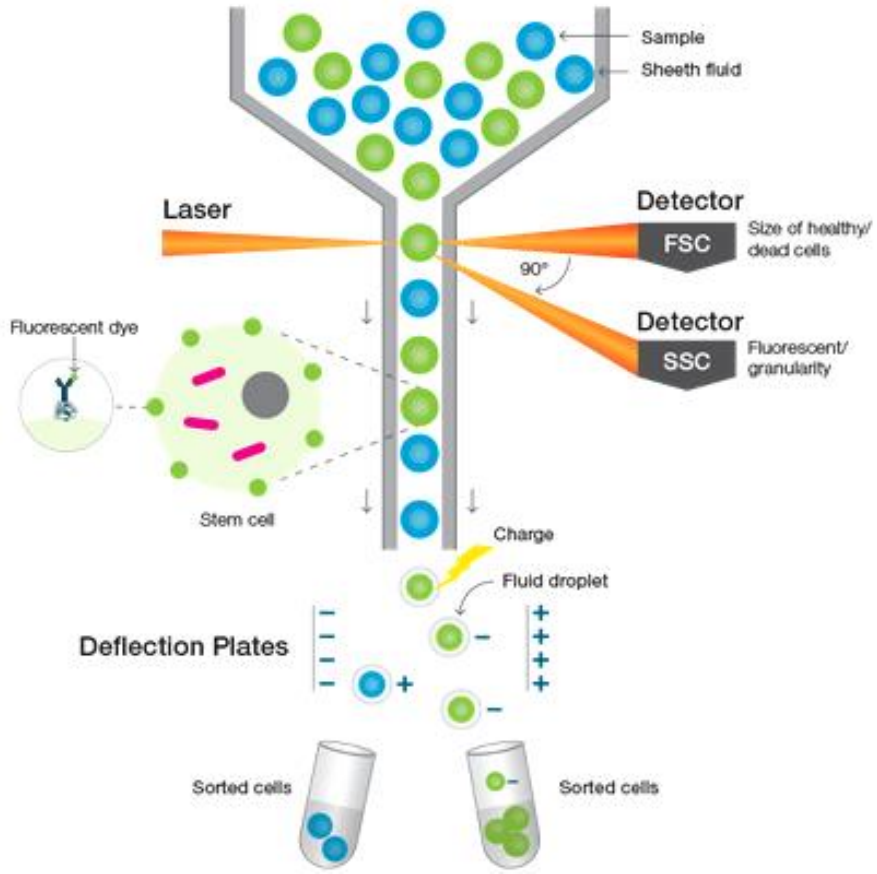


Figure 42. Cell sorting.

4.2.2. Principle

Magnetic cell sorting can be used to isolate a cell type and can also be applied to organelles, proteins, lipids and nucleic acids. In this way, complex mixtures can be processed to select or remove a cell or molecule of choice. Each droplet is analyzed. Depending on the values taken by each parameter, it may be decided to separate this event from the others. Separation is achieved by electrifying the droplet, then deflecting it using electric or magnetic fields. Depending on the equipment, one, two or four populations can be sorted. **Table 6** shows the sorting process as a whole.

Fluorescence labelled cell sorting (FACS) is the gold standard for cell isolation, providing populations that are over 95% pure and virtually free from contamination. Whether your aim is to develop new biologics or pave the way for a better understanding of disease pathophysiology, it is essential to isolate cells with minimal contamination.

Table 6: Characteristics of different sorting techniques

Sorting type	Designation	Advantages	Disadvantages
Mechanicalsorting	Diverts the portion of liquid containing the particle to be sorted	Easy to use, closed system, no aerosol generation	Slow sorting (around 300-500 cells/s max.) due to physical constraints, dilution of sorted cells, mechanical impact on particles

Electrostatic sorting	Pressure from sheath/sample liquid	Very fast (up to 70,000 cells sorted per second), low droplet volume, no mechanical impact on cells, multi-path sorting, cell viability	Expensive, open system (aerosol formation), sensitive to temperature, requires experienced technician for setup
Photodamage sorting	All particles except those of interest are eliminated by a high-energy pulsed laser beam designed to destroy them	/	/

4.2.3. Applications

Sorted samples can be recultivated to create cell lines, for example, to express a particular protein or to be used for metabolism experiments, sequencing, DNA or RNA extraction, re-injection into mice, or microscopy. The cells can be collected in different formats (tubes, multi-well plates).

Cell sorting by FCM, like simple analysis, can be applied to many types of samples as long as they can be put into suspension: yeast, bacteria, murine cells, human cells, fruit fly cells, zebra fish cells, plant cells (nuclei, nucleoli, protoplasts), microalgae, etc.

Cell culture

Generalities

Cell cultures are in vitro cultures of cells, tissues and organs in an artificial medium, i.e. with a known composition and without variations due to metabolism. These recent techniques are linked to the development of biotechnologies. Their aim is to study physiological phenomena and biochemical mechanisms without the need for in vivo experimentation.

Types

5.2.1. Primary cultures

Tissue cellsare dispersed by enzymatic hydrolysis of the protein making up the extracellular matrix. Cell multiplication stops when an element in the nutrient medium is depleted. When they are cultured on a support, their growth stops due to contact inhibition. Explant culture is the oldest type and enabled the precursors of tissue culture to obtain the first cells *in vitro*(**Figure 43**).

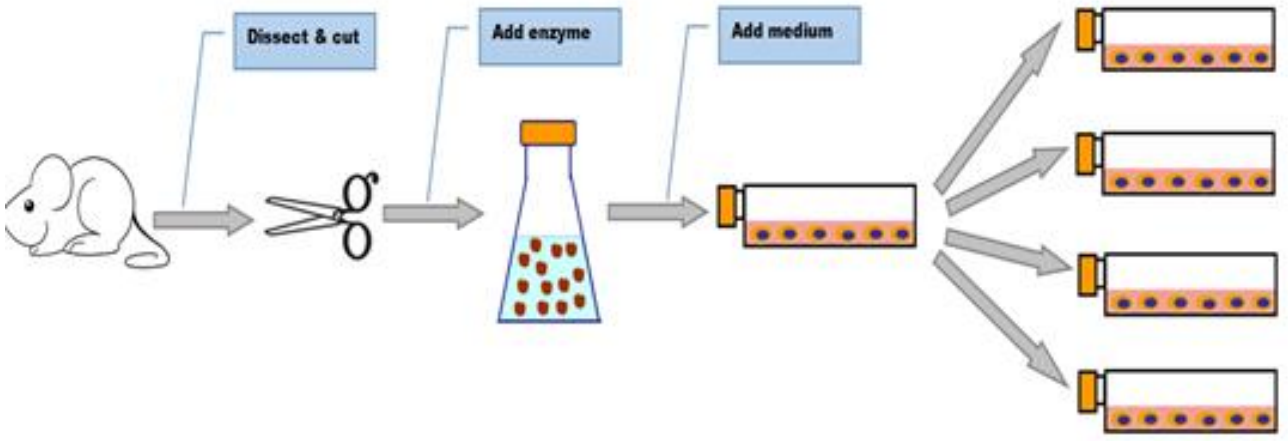


Figure 43. Primarary cell culture.

Mechanical and enzymatic dissociation (**Figure 44**), the most commonly used method, this process is accelerated by adding digestive (proteolytic) enzymes, such as trypsin or collagenase, to tissue fragments to dissolve the cement holding the cells together.

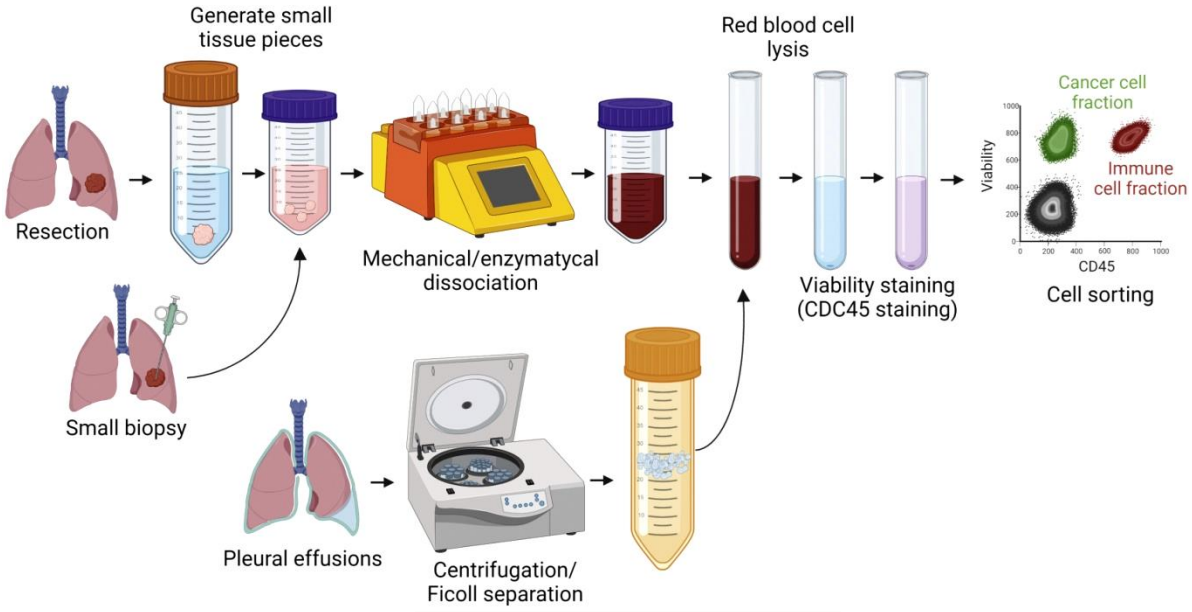


Figure 44. Mechanical and enzymatic dissociation.

5.2.2. Secondary cultures

These are the cells from the primary culture that are used to seed other cultures and so on, leading to secondary cultures. The cells obtained in this manner retain the characteristics of the original tissue, but their number of divisions is limited, as in the organism.

5.2.3. Cell Lines

Occasionally, in a population of cultured cells, one cell acquires characteristics that allow it to divide indefinitely. These transformations can occur spontaneously or be induced (by chemical agents, viruses, etc.). Such cells give rise to immortalized cell lines, which are highly useful as they provide a homogeneous and indefinite cell supply.

Culture Conditions

The conditions for cell culture are as follows:

- **Oxygen:** Animal cells are strictly aerobic, so the supply of oxygen is essential.
- **Temperature:** Cells must be maintained at the temperature corresponding to their organism of origin. Temperatureregulationismandatory(typically at 37°C).
- **pH:** Cells must be kept at the pH level suitable for the organism from which they were derived.
- **Osmolarity:** To prevent an increase in osmolarity due to evaporation, the incubator atmosphere must be saturated with water vapor (humidity at 84-85%).

Origin and collection of cells

There are two types of cells in the organism, as shown in **Figure 45**:

- Circulating or free cells, such as blood cells, are easily collected by differential centrifugation.
- Solid tissue cells, which can be recovered using two methods:
    - Cellular migration from explants.
    - Tissue dissociation with cell release.

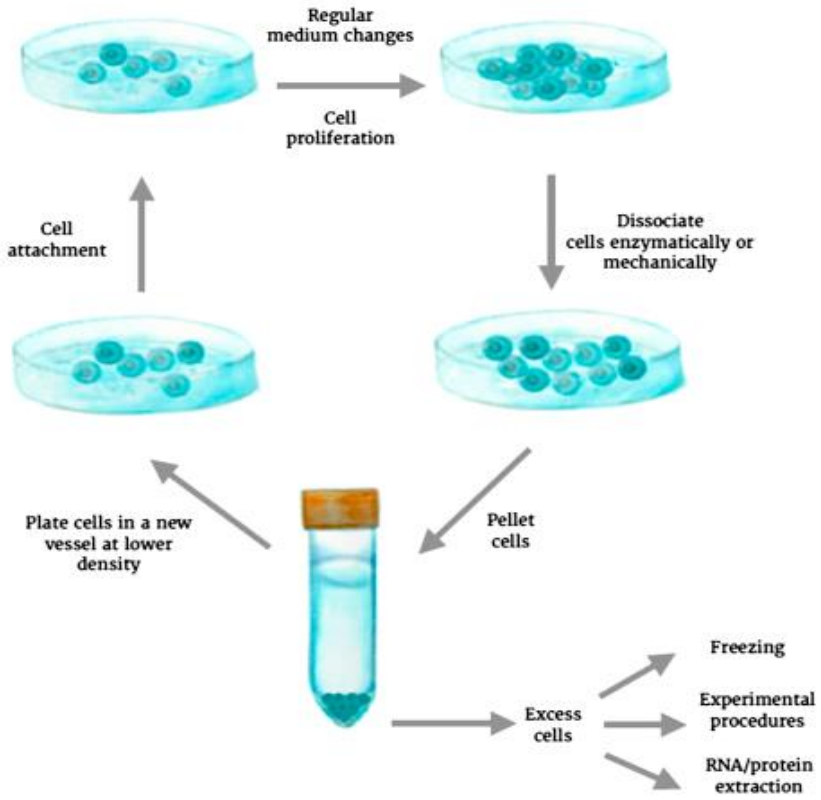


Figure 45. Cell origin and production.

Cell culture evolution

During cell culture, the growth rate of cells is not constant. Instead, it follows a curve with four distinct phases (**Figure 46**):

1. **Adaptation or latency phase**: Due to cell death, there is little to no cell growth as the cells adapt to their new environment and settle.
2. **Exponential growth phase**: Cells divide rapidly as they consume most of the nutrients in the culture medium.
3. **Saturation phase (Confluence)**: The number of cells remains constant because as many cells die as are produced. This is due to nutrient depletion, waste accumulation, and a lack of available space.
4. **Decline phase (Apoptosis)**: Nutrients and space become too scarce to sustain the maximum number of cells, leading to a decrease in the cell population.

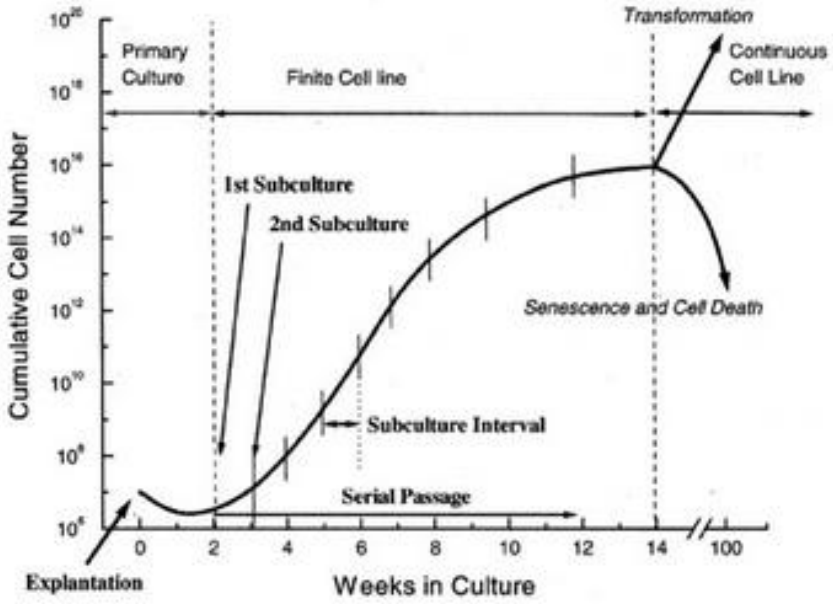


Figure 46. Cell culture growth and subculturing.

5.5.1. Cell confluency

When cells reach confluency, they stop dividing due to contact inhibition. At this point, a passage or subculture is necessary. This involves redistributing the cells into several flasks (**Figure 46**) or discarding a portion and adding fresh medium. This provides the cells with new nutrients and space to adhere.

5.5.2. Passage

Cells can be passaged by scraping the cell layer or, more commonly, using a mixture of trypsin and EDTA (a process known as trypsinization).

- Trypsin is a protease that cleaves peptide bonds at basic amino acids (lysine or arginine), breaking down the proteins involved in cell adhesion, thus detaching the cells from the support. The action of trypsin must be carefully timed to avoid damaging the cell membranes.
- EDTA is a chelator of divalent ions (e.g., Ca^{2+} and Mg^{2+}), which are involved in cell adhesion. Chelation of these ions furtheraids in celldetachment.

The process is monitored under an inverted microscope, with cell morphology changing to spherical as they detach. Trypsin's action is stopped by diluting with fresh medium and neutralized by inhibitors present in fetal bovine serum (FBS).

Culture medium

The culture medium is composed of a basic medium and serum:

- **Medium**:
  Water
  - **Mineral salts**(Na^+, K^+, PO_4^-, Mg^{2+}, Ca^{2+}, Cl^-): These serve as enzyme cofactors, regulate osmotic pressure, membrane potential, transport (e.g., Na^+ cotransport), pH regulation, and aid in cell attachment (especially Ca^{2+}).

- **Amino acids**: Building blocks of proteins. Glutamine (Gln), a key amino acid, is often added as it is unstable.
- **Vitamins**: Essential for various cell types as enzyme cofactors or precursors in molecular synthesis.
- **Glucose**: Provides energy and carbon for catabolic and anabolic processes.
- **Phenol Red**: A pH indicator with a color change range between pH 7.2 and 7.6, allowing visual monitoring of pH shifts.

- **Serum**:
  - Fetal bovine serum (FBS) is commonly used as it promotes cell proliferation by supplying growth factors, adhesion proteins, transport proteins, and trace elements.
  - It enhances buffer capacity and offers protective properties during cell agitation, while also containing protease inhibitors (neutralizing trypsin used in passaging).
  - Antibiotics such as penicillin, streptomycin, and antifungals are added to prevent contamination.

5.5.3. Materials

- **Facilities**: Cell culture requires absolute sterility, as microbial contamination leads to cell lysis. Sterile work environments, such as laminar flow hoods with air filtration systems (PSM), ensure sterility during handling.
- **Support**: For adherent cells, sterile polystyrene containers are used, which are non-toxic and allow for optimal observation under an optical microscope. Adherent cells may also be grown on glass slides or microplates (**Figure 47**), while non-adherent cells grow in suspension under the same conditions (**Figure 48**).

Figure 47. Adherentcell culture plates.

Figure 48. Flasks for suspension cell culture.

5.5.3.1.Incubators for cell culture

Cells are cultivated in incubators set to 37°C (for mammalian cells), with a controlled atmosphere of 5% CO_2. These incubators must be regularly decontaminated to maintain a sterile environment.

5.5.3.2.Contaminants

There are two main types of contamination in cell cultures: chemical and biological.

- **Chemical Contamination**: This is the hardest to detect, as it results from invisible agents like endotoxins, plasticizers, metal ions, or traces of chemical disinfectants. Since these contaminants are not easily visible, they can go unnoticed and affect cell viability and experimental results.
- **Biological Contamination**: This includes fast-growing microorganisms like yeasts, bacteria, and fungi, which are easier to detect as they cause visible changes in the culture (e.g., increased turbidity or changes in medium pH). The presence of antibiotics in the culture medium can sometimes mask these contaminations. However, other forms of biological contamination, such as mycoplasmas and viruses, are more insidious. They do not cause visible changes and often require specific detection methods like PCR or ELISA to identify their presence (**Figure 49**).

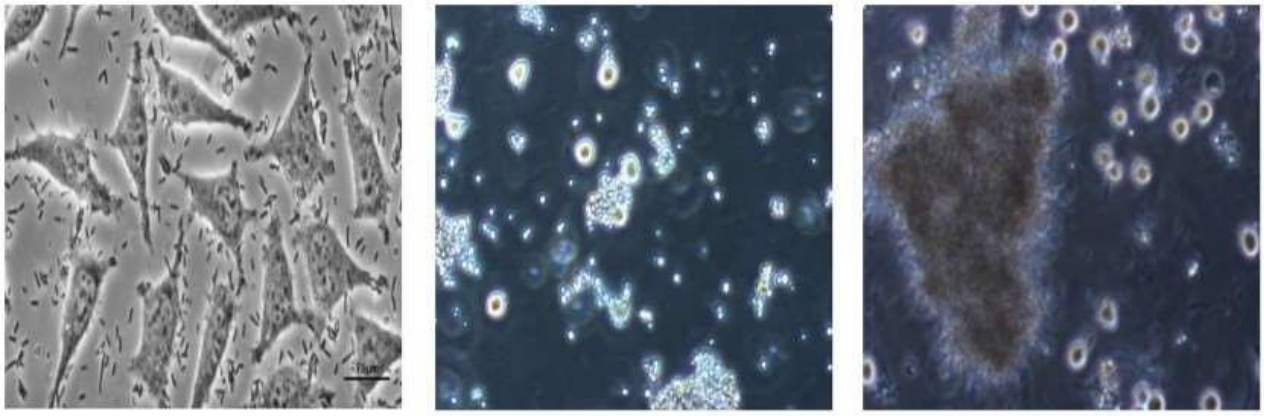


Figure 49. Microbial contamination in mammaliancell cultures.a:bacterial contamination; b: yeast contamination etc: fungal contamination.

5.5.3.3.Cell preservation

- Freezing: Cells are frozen in the presence of a cryoprotectant, such as DMSO (dimethyl sulfoxide). A suspension of about 4 million cells per ml in the exponential growth phase is gradually cooled to -40°C at a rate of 1-4°C per minute and then stored in liquid nitrogen at -196°C.
- Thawing: Cells are thawed quickly at 37°C, followed by a wash to remove DMSO, and then a viability check is performed.
- Viability Check: This involves counting cells in the presence of a dye, such as Funk Blue or Trypan Blue, which can distinguish between live and dead cells under an inverted microscope.

Applications

1. Understanding cellular function: Researchers use cell cultures to study how cells function, grow, and respond to various stimuli.
2. Testing medications and products: Cell cultures are used to test drugs, cosmetics, and the toxicity of chemicals, reducing the need for animal testing.
3. Vaccine production: Certain viruses required for vaccine production are grown in cultured cells, providing essential materials for immunization development.
4. Tissue engineering: Cultured cells are used to grow tissues, such as skin for burn victims, offering regenerative treatment options.

Cytotoxicity and viability test

Cellviability and cytotoxicity

Cell viability refers to the number of healthy cells within a population and is often measured using assays that evaluate enzymatic activity, membrane integrity, ATP production, and other cellular indicators. These methods can utilize luminescence, fluorescence, or colorimetry as indicators of general cell viability, or even assess specific cellular pathways. Cytotoxicity and viability tests are commonly used to assess the impact of drugs or treatments on cells, providing valuable tools in therapeutic research and for enhancing our understanding of cellular function.

6.1.1. Cytotoxicity

Cytotoxicy is a major strategy used by the immune system to fight external threats and can lead to the destruction of cells. It is often evaluated by recording changes in the mitotic index of meristematic cells and is critical for routine monitoring of cellular health and proliferation rates.

6.1.2. Conducting a cytotoxicity test

Biological targets for cytotoxic agents are generally grouped as follows:

- **Cell membranes**: These may undergo lipid peroxidation or lose selective permeability.
- **Mitochondria**: Cytotoxic agents can inhibit oxidative phosphorylation, fatty acid beta-oxidation, cellular respiration, and ultimately reduce ATP concentrations.
- **Lysosomes**: These agents can impair the cell's degradation abilities.
- **Genetic material**: Genotoxic substances can alter the genetic content of cells.

A standard cytotoxicity test consists of three phases:

- **Equilibration**: After inoculating the cells, a stabilization phase allows the cell population to equilibrate before introducing the test drug.
- **Drug exposure**: The drug is introduced, often at various concentrations, to establish a dose-response curve and observe cellular behavior. The exposure duration may vary, with shorter contacts revealing immediate necrotic effects and longer exposures (up to several days) revealing inhibited proliferation.
- **Measurement**: Viability or cytotoxicity measurements are taken post-exposure, using methods such as LDH or ATP measurement (in minutes) or MTS or resazurin assays (over hours). From this data, the **LD50**(lethal dose for 50% of the cells) is determined.

6.1.3. Cytotoxicity analysis

This section focuses solely on ***in vitro***cytotoxicity evaluation methods as alternatives to whole-animal testing (**Table 7**).

Table 7: Comparison of *in vivo* and *in vitro*cytotoxicity tests

Parameters	*In vivo*	*In vitro*
Experimentation	Animal testing, validated by ethics committees	Availability of specific cell cultures
Conditions	Requires a valid animal protocol and proper housing (animal facility)	No animal ethics required, uses specific cell lines
Studytype	Embryonic development, pharmacokinetics, and distribution in tissues	Studies specific cell types
Molecular	Limited to small doses	Allows detailed testing of molecular

6.2. Viability and cytotoxicity assays

Cytotoxicity or viability assays, widely used and recognized for evaluating cell health, include both cell activityandmorphological identification tests. These assays typically count the percentage of live cells based on mitotic indices after exposing a cell culture to a wide range of genotoxic agents.

6.2.1. Dye exclusion assays

6.2.1.1. Trypan Blue Exclusion test

Trypan Blue is a blue dye that selectively interacts with damaged cells. Dead cells are stained blue, while live cells remain unstained due to their intact membranes. The proportion of living and dead cells can be directly counted using a Malassez counting chamber after Trypan blue staining. Cell viability is calculated as follows:

Viability (%)=(Total cells−Blue-stained cellsTotal cells)×100\text{Viability (\%)} = \left(\frac{\text{Total cells} - \text{Blue-stained cells}}{\text{Total cells}}\right) \times 100 Viability (%)=(Total cellsTotal cells−Blue-stained cells)×100

Though this test offers a quick viability check after cell isolation or before seeding, it lacks sufficient sensitivity and reliability.

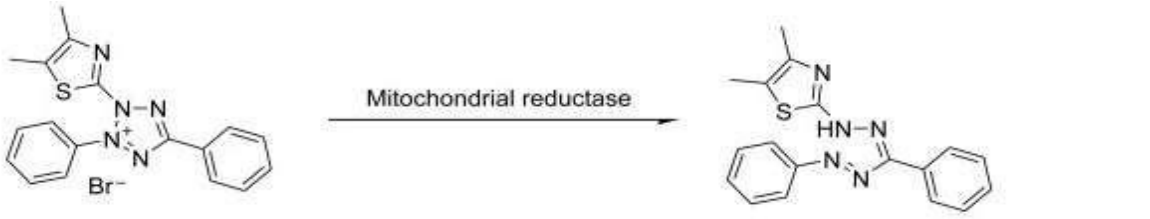
6.2.1.2. Erythrosine B Exclusion test

Erythrosine B, or FD&C Red No. 3**, is a**tetraiodo fluorescein dye used in biological staining and food coloring. Like Trypan Blue, Erythrosine B works on the principle of membrane integrity, staining only dead cells that cannot maintain intact plasma membranes. One key advantage over Trypan Blue is that Erythrosine B is non-toxic, doesn't bind to serum proteins, and requires no incubation before counting.

6.2.1.3. Colorimetric assays

The Neutral Redcytotoxicity assay is commonly used to detect cell viability or drug-induced cytotoxicity. This method is based on the ability of viable cells to absorb and incorporate Neutral Red into their lysosomes. Only viable cells can uptake the dye through active transport, while non-viable cells cannot. The amount of dye retained correlates with the number of living cells, and the results are quantified by measuring absorbance at 540 nm**.**

The MTT assay([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) is a rapid and sensitive method for quantifying cell proliferation and viability. It measures mitochondrial dehydrogenase activity in live cells, which reduces MTT (a yellow substrate) into purple formazan crystals**.** These crystals are indicative of the metabolic activity of viable cells and can be quantified spectrophotometrically (**Figure 50**).



3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide **(MTT)**

(E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenyl-formazan **(formazan)**

Figure 50. Enzymatic reduction of MTT to formazan.

The MTS testwas developed as a newer tetrazolium analogue of MTT for high-throughput screening of in vitro cell growth. MTS, or {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt}, is reduced into a soluble formazan product by mitochondrial dehydrogenase in metabolically active cells, indicating viable cells. The amount of formazan produced is directly proportional to the number of viable cells. This mechanism can be exploited for semi-automated assays to screen compounds with potential anti-leishmanial activity. The MTS test provides a simple, reproducible, and reliable method for evaluating cell viability, making it suitable for drug screening and growth kinetics studies.

The XTT testinvolves the tetrazolium salt 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, which can rapidly penetrate intact cells. This assay detects cell viability based on cellular activity. However, its use for evaluating mature biofilms may yield inaccurate results, as the deeper cell layers of the biofilm tend to become relatively quiescent during the later stages of biofilm formation (**Figure 51**).

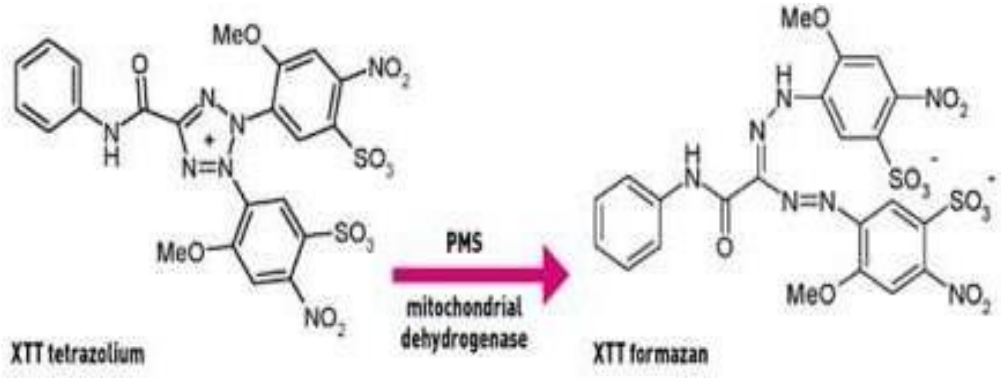


Figure 51.XTT test.

Tetrazolium yellow (XTT) is converted or cleaved by a mitochondrial dehydrogenase into formazan, a soluble orange dye that can be measured by absorbance at 490 (or 450) nm in a microplate reader.

6.2.1.4. Fluormetric assay

Alamar blue (AB)is a faster and more sensitive test than MTT. It is used to assess metabolic function and cellular health. In recent years, the AB test has been widely used in cell viability and cytotoxicity studies for biological and environmental applications, and can also be used to establish the relative cytotoxicity of agents within different chemical classes. Using the REDOX indicator resazurin (oxidised form), it is possible to measure cell proliferation spectrophotometrically. Resazurin is blue and non-fluorescent, while resorufin (reduced form) is red and highly fluorescent. This is a simple, one-step procedure in which metabolic activity leads to the chemical reduction of AB (**Figure 52**).

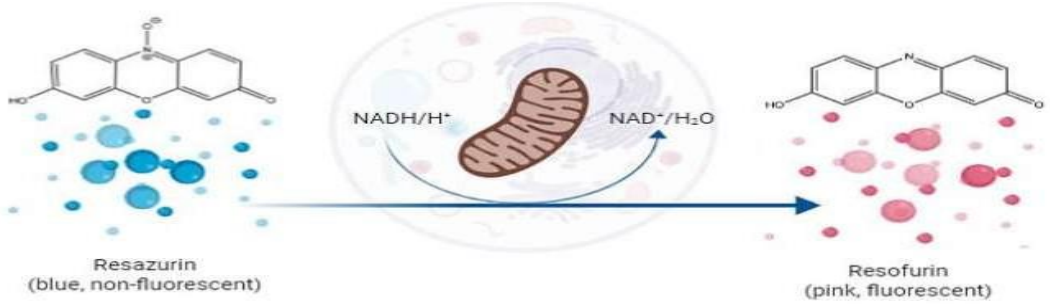


Figure 52. Alamar blue test.

AB is reduced by FMNH2, FADH 2, NAHD, NADPH and cytochromes. Alamar blue is fluorescent and changes colour in response to chemical reduction, and the extent of conversion is a reflection of cell viability.

Fluorescence 5-carboxyfluoresceindiacetateacetoxymethylester5- CFDA-AM is another compound used in fluormetric cell viability assays like Alamar Blue. 5-CFDA-AM is a target of non-specific intracellular esterase enzymes in living cells. Following the non-specific enzymatic activity of esterases, 5-CFDA-AM is converted into a fluorescent substance, carboxyfluorescein, which is polar and non-permeable across the cell membrane of living cells.

6.2.1.5. Measuring membrane integrity

Unlike many other cytoplasmic enzymes that exist in many cells either in low quantities (alkaline and acid phosphatase, for example) or are unstable, LDH lactate dehydrogenase is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant after damage to the plasma membrane. LDH activity can be determined by a coupled enzymatic reaction: LDH oxidises lactate to pyruvate, which then reacts with INT-tetrazolium salt to form formazan (**Figure 53**). The increase in the quantity of formazan produced in the culture supernatant is directly linked to the increase in the number of lysed cells.

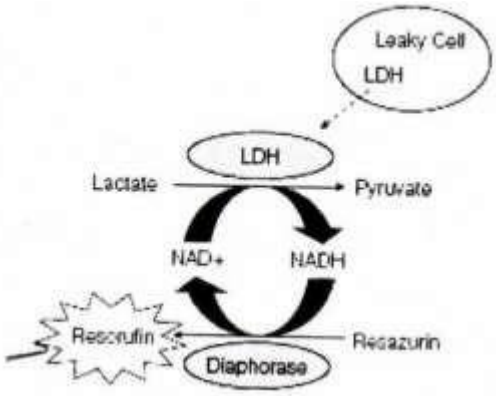


Figure 53.LDH Test.

6.2.1.6. **Limnometric measurements ATP test**

The method uses luciferase, a purified firefly enzyme which catalyses the following reaction:

ATP + d-Luciferin+O2→Oxyluciferin+ AMP+Pyrophosphate + CO2+ lumière (560nm),

The ATP bioluminescence assayis a common technique used to quantify ATP levels and detect living cells. Intracellular ATP is a valid indicator of cell viability. ATP synthesis is interrupted and depleted by ATPases as soon as cells lose their membrane integrity and cell viability. The intracellular concentration of ATP can vary as a function of cellular stress factors and physiological changes such as disease and treatment. To measure ATP, cells must first become permeable to ATP so that the luciferase enzyme can interact with intracellular ATP. Next, the intracellular ATP ases are inactivated and finally the light is measured by luminometers to determine the levels of intracellular ATP (**Figure 54**).

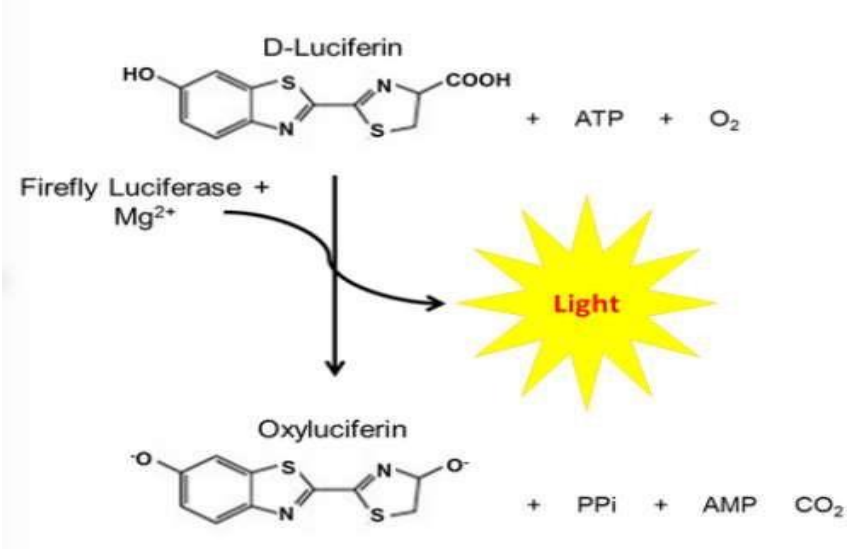


Figure 54.ATP Test

6.2.1.7. **Real-time viability test**

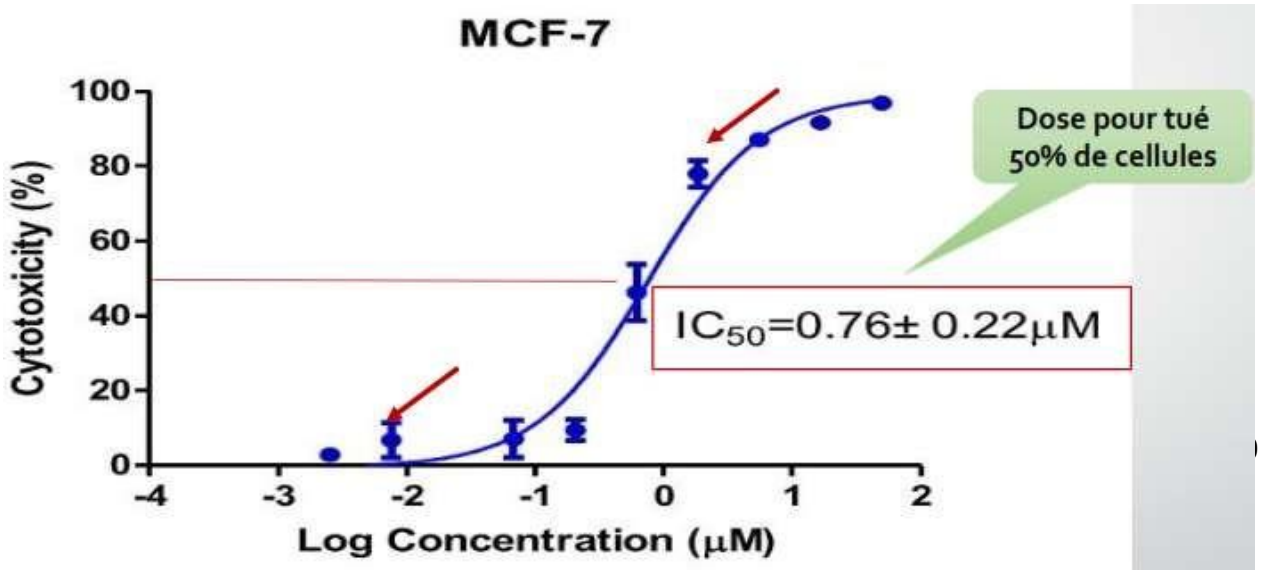
The real-time viabilityassayis the onlycellviabilitymethodthat monitors cellviability in real time. In thismethod, cell-permeable pro-substrate and luciferase are added to the culture medium, but the cells are not lysed to release intracellular ATP. Instead, viable cellstake up thepro-substrate and convertitinto a 'substrate' that diffuses into the culture medium. The luciferase enzyme then uses the diffusedsubstrate to generate a luminescent signal.

6.3. Evaluation of cytotoxicity or viability

These are methodsbasedessentially on disruption of membrane permeability, e.g. cytotoxicity tests usingdyes and othersmeasuring the release of moleculesinto the extracellular medium. Or methodsbased on alterations in cellproliferation(counting and reflecting the number of cells, biochemicalmethodsthatquantify DNA and total proteins). Othermethodswithdifferentprinciples have been used, such as morphologicalmethodsbased on the study of cellular alterations up to lysis.

A mechanistic study involves determining whether the substance to be tested can penetrate the cell by which mode of transport (diffusion, passive or active transport). The next question to askiswhatis the final biologicaltarget of this substance and what dose shouldbechosen (**Figure 55**).

Figure 55. Dose- and time-dependent curve. The acute toxic effect was observed after 24h, 48h, 72h, and 96h.



This table summarizes the main cytotoxicity and cell viability tests, highlighting their respective principles, indicators and measurement methods.

Table 8: Main cytotoxicity or cell viability tests

Technique	**Trypan Blue**	**Neutral Red**	**MTT**	**Resazurin**
Test	Viability	Viability	Metabolic capacity	Metabolic capacity
Principle	Dye exclusion, only dead cells are stained	Neutral red for live cells (inclusion dye)	MTT metabolized from yellow to violet formazan precipitate	Fluorescence or color change, reduced irreversibly to resorufin, a highly fluorescent pink/red indicator
Reading method	Cell counting with hemocytometer under microscope	Absorbance reading at 540 nm	Absorbance reading at 570 nm or 600 nm	Fluorescence (Excitation at 530 nm, Emission at 590 nm) or absorbance at 570 nm

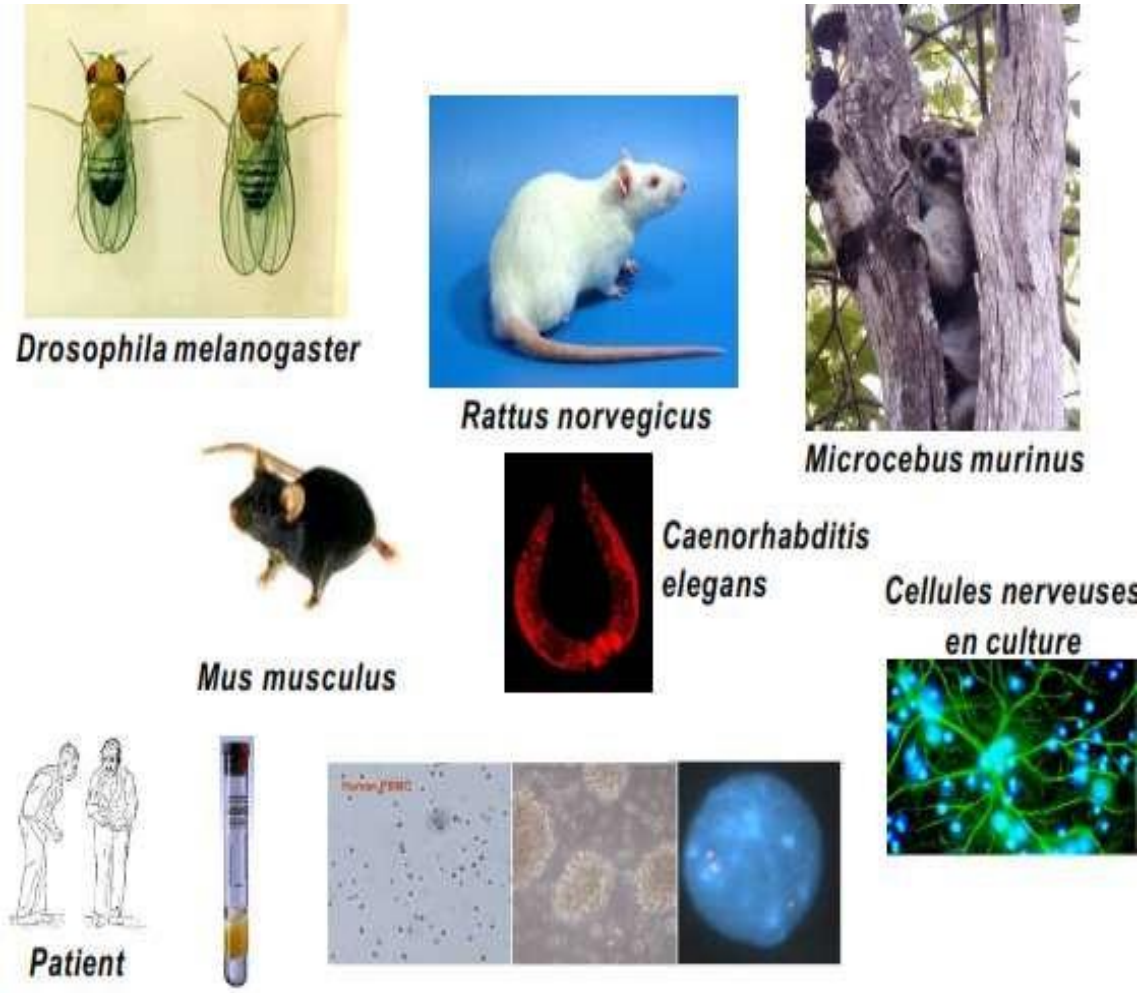
Cell models, tissue models and animal models

Most often, the presentation of scientific results is done without placing the entire framework of the experiment into context. Data ispresentedwithoutmentioning the animal model(s) used, whichis a seriousoversight, as wewillattempt to demonstrate in this contribution (Figure 56).

The different types of modelsinclude:

- Molecularmodels (test tubes),
- *In vitro*/*ex vivo*models (cell cultures, immortalized and transformedcelllines, organ cultures, and tissue cultures),
- *In vivo*models,
- *In silico*models.

Figure 56. Different types of experimental model.



Cellular and tissue models

7.1.1. Stem Cells

Stem cells possess the properties of self-renewal and differentiation into various cell types (**Figure 57**). As such, they present a promising alternative to primary cultures. Stem cells can be of embryonic origin, extracted from a blastocyst (within the first seven days of the embryo's development), which raises ethical concerns. Pluripotent stem cells can also be harvested from umbilical cords. Another technology has been developed to enable somatic cells taken from an adult to become stem cells: induced pluripotent stem cells (iPS).

The choice of cell typedepends on the objective of the experiments. In the context of toxicological screening studies (involving a large number of compounds), cell lines appear to be the fastest and most cost-effective method to characterize toxicity, based on a series of criteria targeting key cellular functions. These tests can be automated for high-throughput screening.

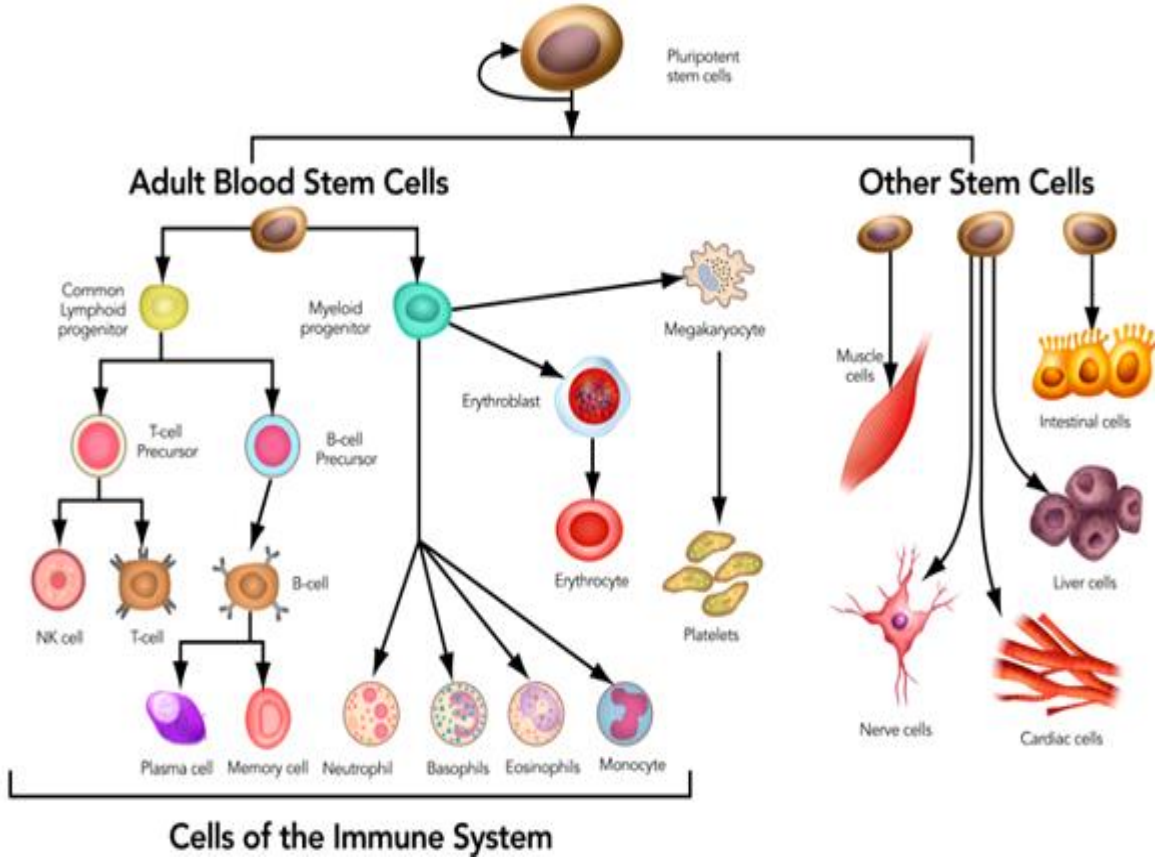


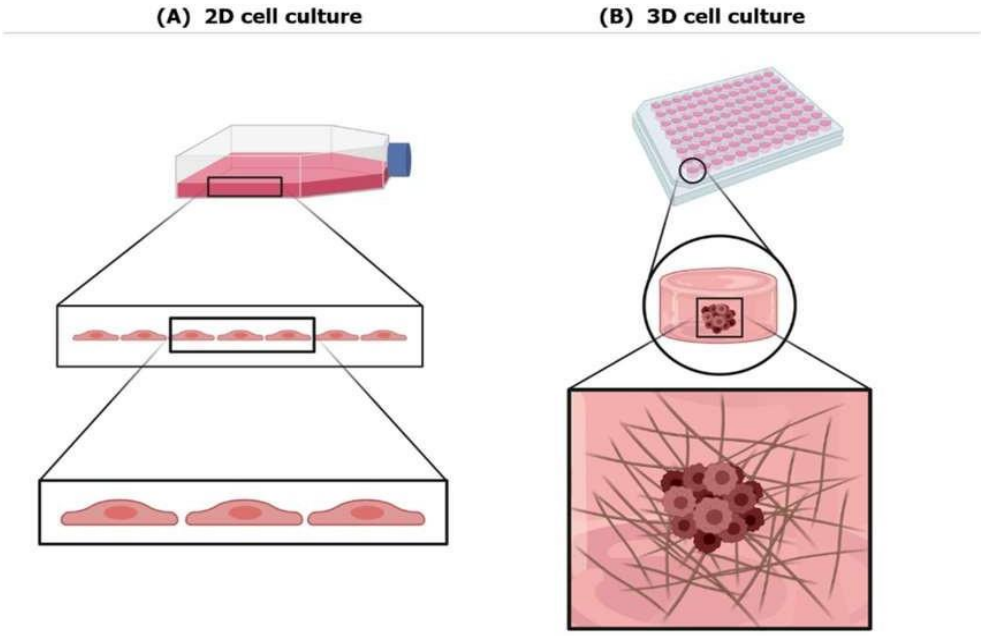
Figure 57. Stem cell renewal and differentiation.

2D and 3D Cell Cultures (2D Model and 3D Model)

Two-dimensional (2D) cellculture is the oldest and most common method: cells adhere to a flat surface (e.g., a Petri dish). This surface provides mechanical support to the cells, and the monolayer arrangement ensures that each cell benefits from an optimal amount of nutrients and growth factors (**Figure 58**). As a result, cell proliferation is homogeneous.

These culture methods are adaptable to robotics and automation. However, most 2D methods do not allow control over the morphology, orientation, or shape of the cells. Three-dimensional (3D) cell culture, on the other hand, aims to model the *in vivo* interactions of tissues in order to study biochemical and biomechanical signals. A well-designed microenvironment in cell and tissue engineering can be used to promote cell proliferation, migration, matrix production, and stem cell differentiation. These three-dimensional systems can be based on different supports: macroporous and microporous networks (composed of polymers or extracellular matrix proteins), hydrogels (syntheticor natural), or plastic supports (inserts or microstructured films)

Figure 58. Thematic organization differences in between 2D and 3D cell cultures



7.2.1. Pheroids

Spheroids are cell aggregates that adhere to each other through extracellular matrix and intercellular connections. They are more complex than 2D cultures due to cell-cell and cell-matrix interactions, providing a better *in vitro* model of the microenvironment of tissues *in vivo*.

Spheroids are one of the most common and versatile methods for cultivating cells in three dimensions. There are several methods to obtain spheroids from tumor cells or primary cells, including:

Microfluidicstechniqueensures a continuous supply of oxygen and growth factors during culture by using materials permeable to soluble factors (**Figure 59**).

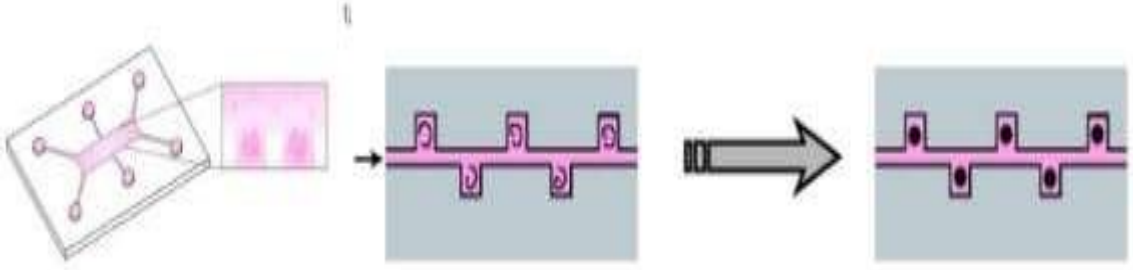


Figure 59. Diagram of pheroids coupled with microfluidic technology.

Micropuces

PDMS wells with pyramid-shaped openings are formed to encourage the formation and growth of the spheroids and to control their size. These techniques are known as spheroids-on-a-chip (*Spheroidsona chip*).

7.2.2. Embryoid bodies

From pluripotent cells (embryonic stem cells or pluripotent stemcells). Pluripotent stem cellstransformintospheroidswithluminal structures (**Figure 60**). Such self-organisation recapitulatesthe processes of earlyembryogenesis. Aggregates of induced pluripotent stem cellsformembryoid bodies consisting of outercells, whichdifferentiatetowards the endodermalepithelium and deposit a basement membrane. Adjacent epiblasticcells polarise into a columnarepithelium.

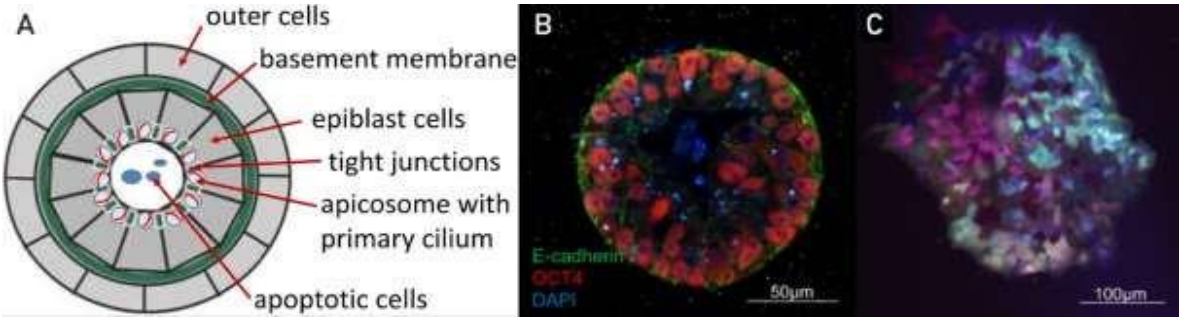


Figure 60. Embryoid bodies.

7.2.3. Organoid structure

These structures, called organoids, are obtained from adult stem cells, under culture conditions (growth factors, extracellular matrix) that need to be adapted for each type of organ studied. By multiplying, the cells generate several cell types like theirin vivo equivalent and recreate a three-dimensional (3d) organisation similar to their original tissue. Organoids contain several epithelial cell types representative of their tissue of origin, but cells of mesenchymal, stromal, immune and neural origin are absent.

Unlike traditional cultures, organoids are formed from stem cells, the cells of which they are composed are genetically normal and their genome is very stable (**Figure 61**). Recently, several teams have begun adding cell types present in the tissue microenvironment (fibroblasts, immune cells) to organoids. The ultimate aim is to be able to use well-defined models to study in vitro cellular pathophysiologies similar to those observed *in vivo*.

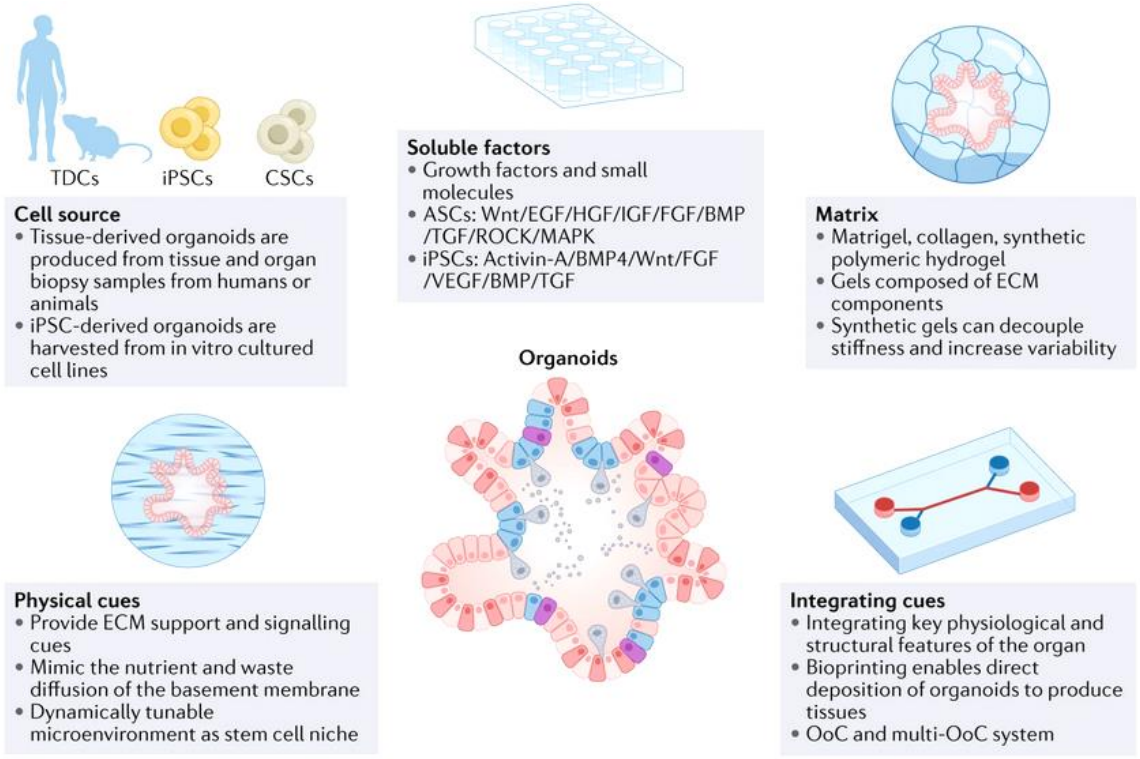


Figure 61. Organoid structure.

The establishment of organoid-based culture requires considerations about major components that make up organoid cultures cells, soluble factors and matrix, physical cues and the successful integration of these components. ASCs, adult stem cells; CSCs, cancer stem cells; ECM, extracellular matrix; FGF, fibroblast growth factor; iPSCs, induced pluripotent stem cells; OoC, organ-on-a-chip; TDCs, tissue-derived cells.

Animal models

7.3.1. Description

An animal model is a non-human animal with a condition similar to a human condition and used as a model for the study of that condition. In biomedical research, an animal model is a model which allows the study of reference data on biology or behaviour, or in which a spontaneous or induced pathological process can be studied, which has one or more aspects in common with an equivalent phenomenon in humans or other animal species (**Figure 62**).

The areas of use can be grouped into:

- Pure research consists of fundamental research and the acquisition of new knowledge e.g. Mutagenesis,
- Applied research consists of research in the medical field, in particular the search for new therapies e.g. allergies, toxicity,
- Teaching involves the transmission of knowledge to students,
- Biotechnology for the production of poly and monoclonal antibodies.

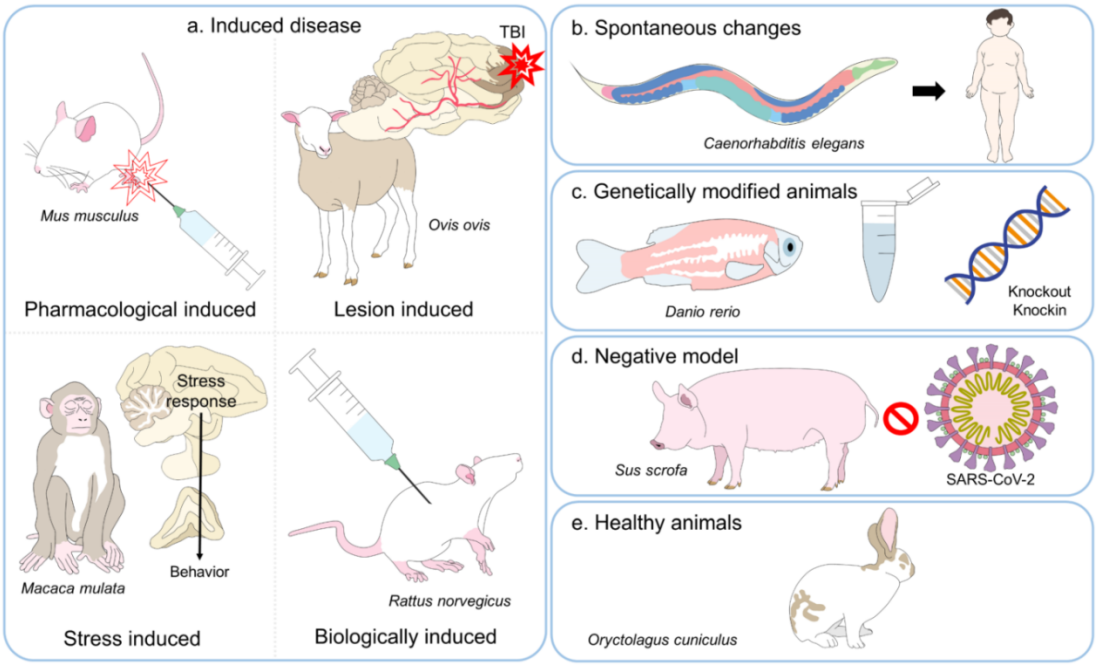


Figure 62. Animal models.

7.3.2. Types

The mouse is the most commonly used animal in experimentation due to its advantages, such as ease of breeding, its size, which allows for a large number to be housed in a small space, low-cost nutritional requirements, and its short life cycle with high fertility, enabling large litters. Besides the mouse, other rodents like rats and rabbits are also widely used. Cats and dogs are often employed in veterinary medicine, while monkeys, pigs, and livestock such as cattle and sheep are used in agricultural research, particularly in improving zootechnical parameters (reproduction, feeding, and growth parameters).

There are different types of animal models, as shown in **Table 9**.

Table 9: Different types of animal models

Model	Designation	Examples
Natural wild	Animals with no modifications or manipulation	Diabetes, arthritis, hypertension
Experimental	Induced pathology	Diabetes mellitus, cancer
Genetically modified	Transgenic animals via insertion of foreign DNA or gene knockout	"Oncomice" (cancer research mice), knockout strains
Negative	Animals resistant to certain diseases	Antibiotic resistance
Orphan	Animals with spontaneous diseases	Scrapie in sheep

7.3.3. Genetic Strains

A strain is a group of very homogeneous individuals, obtained through inbreeding, and are as genetically identical as possible.

7.3.3.1. Mice

The mouse (***Mus musculus***)is the most widely used animal in research, accounting for approximately **60%**of all laboratory animals. Mice have undergone various genetic manipulations; resulting in many strains that cater to specific research needs. The most commonly used strains in fundamental biological and medical research include:

- **BALB/c**: This is the albino laboratory mouse, derived from the domestic mouse through a mutation in the gene responsible for melanin production.
- **Nude Mouse**: This hairless "nude" mouse strain is characterized by an underdeveloped or absent thymus. It is primarily used in cancer research due to its lack of T lymphocytes, which allows for the growth of pure tumors without contamination by other cells.
- **C57BL/6**or "Black-6": This black mouse strain is versatile and commonly used in diabetes and obesity research. It serves as the genetic background for the development of transgenic models.

7.3.3.2. The laboratory rat

The laboratory rat is the second most commonly used species after the mouse. *Rattus norvegicus*is the domestic rat from which all other strains have been derived to meet the specific needs of various studies.

The following strains are notable:

1. Albino Wistar rat: The most widespread strain of laboratory rats, from which other strains have developed. It is used in biological and medical research, characterized by a broad head, long ears, and a tail shorter than its body.
2. Long-Evans rat: A strain developed by crossing a Wistar female with a wild gray male, named after Dr. Long Evans. It is used as a model for research on obesity and behavior.
3. Sprague-Dawley rat: A strain developed from the Wistar rat, widely used in medical research, known for its calmness and docility.
4. Zucker rat: An obese strain with a mutation in the leptin receptor (a hormone controlling satiety). It is the standard model for research on obesity and hypertension.
5. Hairless rat: A naturally hairless rat due to a non-induced mutation. Like the nude mouse, the Hairless rat lacks a thymus, which severely compromises its immune system.
6. Brattleboro rat: A strain with a natural mutation in the gene that encodes the antidiuretic hormone "vasopressin," disrupting water and mineral balance, leading to diabetes insipidus, characterized by excessive urination (polyuria) and excessive thirst (polydipsia).
7. Knockout rat ("KO Rat"): A strain where one or more genes are inactivated to mimic human diseases. This model is used to study the function of specific genes and the discovery of appropriate medications.

8. Spontaneously hypertensive rat (SHR): A strain that serves as a model for natural hypertension.

7.3.3.4. Transgenic strains

Transgenic strains refer to animals that have received a fragment of DNA from another species in their genome, or those in which one or more genes have been inactivated. Examples include:

- Doogie mouse: This strain has superior intelligence due to enhanced activity of the NMDA (N-Methyl-D-Aspartate) receptors in the brain.
- Oncomouse: A mouse that has received a human oncogene, making it predisposed to developing cancer.
- Knockout mouse: This category includes all mice that have one or more genes inactivated.
- Cold-tolerant mouse: This mouse is extremely sensitive to cold due to the absence of a sodium channel.
- Fat mouse: An obese mouse predisposed to developing diabetes due to the inactivation of the gene encoding the enzyme carboxypeptidase E.
- Mdx mouse: A model used for studying Duchenne muscular dystrophy, as it lacks the gene that codes for dystrophin.

Choosing the right animal model

All living beings share biological similarities, whether morphological, genetic, biochemical, or physiological, allowing animals to be used as models for human studies. Much of our knowledge in biochemistry, physiology, and pharmacology has been acquired through the study of animal models, which could not have been ethically or religiously experimented on humans.

Choosing the most appropriate animal model for the condition under study is both delicate and essential to validate the extrapolation that will later be made to humans. Animal models used for experimental studies on humans can be divided into different groups:

1. Natural or "spontaneous" models: Animals that naturally present a condition similar to a human condition.
2. Experimental models: Animals in which researchers experimentally reproduce a condition.
3. Genetically modified models: Animals in which the genetic code has been altered to induce the condition under study. To modify the genome of these animals, foreign DNA can be inserted, or specific genes can be replaced or neutralized (knockout models).
4. Negative models: Animals that show resistance to a given condition, and their study helps understand the causes and physiological basis of disease resistance.
5. Orphan models: Animals that naturally exhibit conditions that do not have a human equivalent.

Ethics and legislation in animal research

Ethics committees for animal experimentation (ECAE) were established to address the framework of animal use without harming their well-being. These committees are considered

competent authorities that have the right to ethically evaluate research projects involving the use of animals.

The ECAEis composed of at least five individuals: a researcher, a veterinarian, an experimenter, an animal technician, and a person from the social sector not involved in research activities.

The committee ensures that a validated protocol is followed, ensuring that the use of animals is indispensable without causing unnecessary pain and suffering that could compromise the expected results. This is the principle of cruelty-free science.

In 1959, William Russell and Rex Burch introduced the 3R Rule, which consists of three concepts:

1. Replacement: Seeking alternatives to animal use to reduce their suffering during experimentation.
2. Reduction: Minimizing the number of animals used without compromising the reliability and statistical relevance of the results.
3. Refinement: Reducing to the maximum possible extent the constraints imposed on animals, minimizing their pain, suffering, and anxiety.

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