1. Specific course objectives

1) Proficiency in microscopic observation techniques;

2) Cell fractionation techniques;

3) Cytological and histological methods

2. Biological dimensions and order of magnitude

Each biological scale requires specific tools for its observation and analysis, ranging from electron microscopes to medical imaging techniques.

Tableau1 : Summary of biological orders of magnitude:

Structure	Taille approximative	Unité
Atom	0,1 nm	Nanometer
DNA (diamter)	2 nm	Nanometer
Ribosome	20-30 nm	Nanometer
Virus	50-200 nm	Nanometer
Bacteria	1-5 µm	Micrometer
Animale Cell	10-30 µm	Micromete
Plant Cell	30-100 µm	Micrometer
Epithelial tissue	100 µm - several mm	Millimeter
Organe	10-30 cm	Centimeter
Human Organism	1,7 m	Meter

3. Concept of separating power

Resolving power (or resolution) refers to the ability of an optical system, such as a microscope, to distinguish two nearby points as distinct from one another.

1. Resolving power formula (Rayleigh criterion)

The resolving power depends primarily on the wavelength of the light used (**λ**) and the numerical aperture (**NA**) of the optical system:

d= λ / 2 NA , avec **d** : Minimum distinguishable distance between two points (in nanometers, nm). λ: Wavelength of the light used (in nm). **NA** (**N**umerical Aperture): Depends on the beam angle and the refractive index of the medium (**n**).

NA=n × sin(θ) avec : **n** : Refractive index of the medium between the lens and the observed object. **θ**: Half-angle of the light beam captured by the lens

2. Practical interpretation:

- ✓ The smaller λ is (e.g., blue light rather than red light), the higher the resolving power
- ✓ The larger the numerical aperture (NA), the better the resolution.

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- ✓ The **electron microscope** has better resolving power than an **optical microscope** due to the short wavelength of electrons.

3. Examples of separating powers:

- ✓ Human eye: ≈ 0,2 mm
- ✓ Classical optical microscope: ≈ 200 nm (0,2µm) (limited by the wavelength of visible light).
- ✓ Transmission Electron Microscope (TEM) : ≈ 0,2 nm

4. Operating principle of the bright-field photon microscope

The **bright-field light** microscope (or conventional optical microscope) is one of the most commonly used techniques in biology for observing cellular and tissue structures. It uses visible light to form a **magnified image** of the sample.

1. General principle:

A light source emits **a beam of white light** that passes through the sample. The sample, placed on a microscope slide, is traversed by the light. The objective lens collects the light rays that have passed through the sample and focuses them to form a real, magnified image. The eyepiece acts as an additional magnifying glass, producing a magnified virtual image that the observer can view directly with their eye or capture with a camera.

2. Bright-field observation:

The sample appears against a bright, clear background. Transparent samples often require staining (e.g., Gram stain, hematoxylin-eosin stain).

- ➢ **Advantages** :
  - ✓ Simple and economical technique.
  - ✓ Allows observation of live or fixed samples.
  - ✓ Good resolution for structures larger than 200 nm.
- ➢ **Disadvantages** :
  - ✓ Low contrast for unstained or transparent samples.
  - ✓ Resolving power limited to approximately 200 nm (the diffraction limit of visible light).
  - ✓ Inability to observe fine subcellular structures (e.g., ribosomes).

3. Applications of the bright-field microscope:

- ✓ Observation of plant and animal cells.
- ✓ Analysis of stained histological sections.
- ✓ Studies of microorganisms (bacteria, yeasts).
- ✓ Observation of live preparations (e.g., protozoan movements).

5. The preparatory steps of a sample for observation under a bright-field microscope (the histological technique)

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1. Sample collection

- ✓ **Objective:** To obtain a representative sample of the tissue or organ to be studied.
- ✓ **Methods:** Biopsy, autopsy, surgery, or direct sampling.
- ✓ **Precaution:** The sample must be handled carefully to avoid any damage.

2. Sample fixation :

- ✓ **Objectif** : Preserve cellular and tissue structure, stop enzymatic processes and prevent degradation by microorganisms.
- ✓ **Fixateurs courants** :
  - ❖ **Formol** (formaldehyde) : Universal Fixer.
  - ❖ **Glutaraldehyde** : Fast and efficient fixation for electron microscopy.
  - ❖ **Ethyl alcohol**: Fast fixing but can lead to excessive dehydration.

3. Inclusion :

- ✓ **Objectif** : Harden the tissu to facilitate fine cutting with a microtome..
- ✓ **Techniques d'inclusion** :
  - ❖ **Paraffin embedding:** The most common method, used after complete dehydration.
  - ❖ **Plastic resin embedding:** For very thin sections.
- ✓ **Paraffin embedding steps**:
  - ❖ **Deshydratation**: The water is removed by baths of alcohol of increasing concentration..
  - ❖ **Clarification**: Use of a solvent (e.g., xylene) to replace alcohol and prepare for infiltration.
  - ❖ **Infiltration** : Impregnation of the tissu with liquid paraffin.
  - ❖ **Embedding**: The tissu is placed in a solid paraffin block

4. Preparation of histologicals sections :

- ✓ **Objectif** : Obtaining extremely thin and regular sections (between 3 and 10 µm for optical microscopy)
- ✓ **Tool used** : **A microtome** is a device used to make thin sections**.**
- ✓ **Technique** : The resulting sections are carefully placed on glass slides.

5. Coloring :

- ✓ **Objectif** : Highlight the cellular and tissue structures, as biological tissues are often transparent.
- ✓ **Principe** : Dyes bind specifically to certain cellular structures.
- ✓ **Common techniques**:
  - ❖ **Hematoxyline-Eosine** (H&E): The most commonly used stains the nuclei blue-violet (hematoxylin) and the cytoplasm pink (eosin).

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- **Coloration spéciale** : Trichrome de Masson, PAS (Acide Périodique-Schiff).
- **Specific staining for particular structures**: e.g. labeling of lipids or proteins.

6. Mounting and observation under the microscope:

The slide is mounted between a slide and a coverslip (placed carefully on the sample to avoid air bubbles), then observation.

- **Adjusted parameters**:
  - Light intensity.
  - Diaphragm and condenser.
  - Objectifs (×4, ×10, ×40, ×100).
  - Analyse : Identification of structures labeled by dyes.

7. Interpretation and analysis of results

- Comparison with reference histological sections.
- Identification of pathological alterations or specific structures

6. Operating principle of the fluorescence microscope.

A fluorescence microscope is a type of photonic microscope that uses the phenomenon of fluorescence to visualize specific structures within a cell or tissue. It allows the detection of naturally fluorescent molecules (autofluorescence) or molecules labeled with fluorochromes (fluorescent dyes).

1. Principle of fluorescence

- **Fluorescence**: This is the emission of light by a molecule after absorbing light energy at a specific wavelength.
- **Excitation**: A fluorochrome absorbs light at a short wavelength (e.g., ultraviolet or blue).
- **Emission**: The fluorochrome re-emits light at a longer wavelength (e.g., green, red).
- **Example** :
  - **Excitation** : Ultraviolette Light (UV).
  - **Emission** : Green light (ex. fluorochrome FITC).

2. Applications du microscope à fluorescence

- **Cell Biology**: Study of cell organization and protein interactions.
- **Medical Diagnostics**: Detection of pathogens (e.g., Mycobacterium tuberculosis by immunofluorescence).
- **Genetics**: DNA and RNA labeling.
- **Cancer Research**: Identification of tumor biomarkers.

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- ✓ **Live Cell Observation**: Dynamic monitoring of proteins in living cells.

3. Définition d'un fluorochrome

A **fluorochrome** is a fluorescent molecule capable of absorbing light at a certain wavelength (excitation light) and re-emitting that light at a longer wavelength (emission light). This process is called fluorescence.

- ✓ **Types of fluorochromes** :
- ❖ **Natural Fluorochromes** : Some molecules exhibit natural autofluorescence.
- ❖ **Example** : Chlorophylle, vitamine A, collagène.
- ❖ **Synthetic fluorochromes** Designed to bind specifically to certain molecules.

Examples : **FITC** (**f**luorescéine **I**so**T**hio**C**yanate), Texas Red, Alexa Fluor, Rhodamine.

- ❖ **Protéines fluorescentes** : Genetically modified proteins that emit fluorescence when expressed in an organism or cell. **Example** : **GFP** (***G**reen **F**luorescent **P**rotein*), **YFP** (***Y**ellow **F**luorescent **P**rotein*).

4. Exemple de fluoromarqueurs utilisés (Rhodamine, Fluorescéine...)

Tableau2 : Summary of common fluoromarkers

Fluoromarqueur	Longueur d'onde d'excitation (nm)	Longueur d'onde d'émission (nm)	Application principale
FITC	495	519	Antibody / protein labeling
Rhodamine (TRITC)	550	570	Cytoskeleton labeling
DAPI	358	461	DNA labeling
Texas Red	595	615	protein labeling
Alexa Fluor 488	495	519	Immunofluorescence
GFP	488	507	in vivo protein observation
Cy3	550	570	in situ hybridization
Hoechst 33342	350	461	Live-cell DNA labeling

5. Example of a cellular compound detectable by fluorescence microscopy.

The choice of fluorochrome depends on the type of compound, its location, and the specific objective of the study.

a). L'immunofluorescence ou d'immuno-marquage

Fluorescent molecules (fluorochrome) are linked to Antibodies (AB) which will bind specifically to targeted molecules, Antigens (AG), according to the principle of the AB-AG immune reaction, which facilitates the detection of the AB-AG complex.

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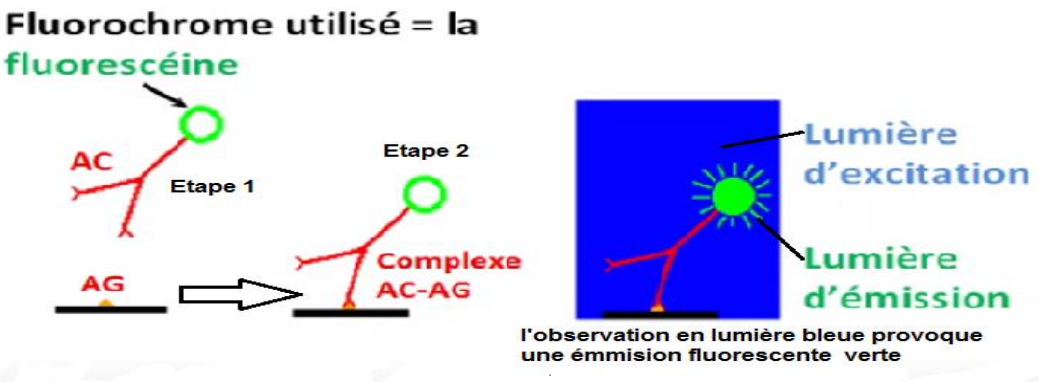

b). Detectable compounds and associated fluorochromes

Tableau 3 : Summary of detectable compounds and associated fluorochromes

Composé Ciblé	Fluorochrome Exemple	Couleur d'émission	Application
ADN	DAPI, Hoechst	Bleu	Cell cycle
Protéines	FITC, Rhodamine	Vert, Rouge	Immunofluorescence
Actine	Phalloïdine-FITC	Vert	Cytoskeleton
Mitochondries	MitoTracker	Rouge, Vert	Energy metabolism
Membrane	DiI, FM 4-64	Rouge	Membrane transport
Calcium (Ca^{2+})	Fluo-4, Fura-2	Vert, Violet	Cellular signaling
pH	SNARF-1	Rouge	Cellular homeostasis

Phase Contrast Microscope: This is an optical microscope useful for observing unstained living cells.

7. Operating principle of the Transmission Electron Microscope (TEM).

1. Principe Fondamental

- ✓ An electron beam is generated by an electron gun under high vacuum.
- ✓ The electrons are accelerated by a high voltage (typically between 80 kV and 300 kV).
- ✓ The beam is focused by a series of electrostatic and electromagnetic lenses.
- ✓ The electrons pass through the ultrathin sample (70–100 nm thick).
- ✓ Dense regions of the sample absorb or scatter the electrons, while less dense regions allow them to pass through.
- ✓ The transmitted electrons form a high-contrast image on a fluorescent screen or a digital detector.

2. Field of application of the TEM

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- ✓ Study of cellular organelles (mitochondria, nuclei, ribosomes).
- ✓ Observation of viruses and bacteria.
- ✓ Analysis of proteins and macromolecules.
- ✓ Visualization of cell interactions.
- ✓ Research in nanotechnology and advanced materials.

3. Sample Preparation for TEM

- ✓ **Fixation**: Use of fixatives such as glutaraldehyde.
- ✓ **Embedding**: The sample is embedded in a resin to harden it.
- ✓ **Ultra-thin sectioning**: Performed with an ultramicrotome.
- ✓ **Contrast enhancement**: Addition of heavy metals (lead, uranium) to increase contrast.
- ✓ Deposition on a stable support for observation (a metal grid).

4. Les différents procédés de contraste électronique (coloration positive, coloration négative, ombrage, autoradiographie)

a). Positive Staining

- ✓ **Principle**: Positive staining involves selectively fixing and labeling biological structures with heavy metals (e.g., **uranyl acetate, lead citrate**). The metal ions bind preferentially to cellular components, increasing their electron density. The stained regions appear dark in the final image because they scatter or absorb more electrons.
- ✓ **Applications**: Visualization of cell membranes. Study of intracellular organelles such as mitochondria and lysosomes.
  - ❖ **Example of a staining agent**:
- ✓ **Uranyl acetate**: Staines structures rich in phospholipids and proteins.
- ✓ **Lead citrate**: Enhances the contrast of biological membranes.

b). Negative Staining

- ✓ **Principle**: Unlike positive staining, negative staining does not directly stain the sample. A solution of heavy metal (e.g., uranyl acetate, phosphotungstate) is applied around the sample. Electrons pass more easily through the sample but are blocked by the surrounding metals, creating a light outline against a dark background.
- ✓ **Applications**: Observation of very small and delicate structures, such as viruses, ribosomes, protein fibers, and the study of isolated macromolecules.
- ✓ **Example of a staining agent**: Uranyl acetate, potassium phosphotungstate.

c). Ombrage Métallique (Shadowing)

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- ✓ **Principle**: Shadowing involves depositing a thin layer of heavy metal (e.g., platinum, gold) onto the sample at a precise angle. This creates a shadow effect on a portion of the sample, enhancing its three-dimensional relief and contrast. An additional layer of carbon is sometimes added to stabilize the sample.
- ✓ **Applications**: Visualization of macromolecules (e.g., DNA, proteins). Study of surface structures. Observation of viruses and subcellular particles.
- ✓ **Materials used**: Platinum, Gold, Carbon (protective layer).

d). Autoradiography

- ✓ **Principle**: Autoradiography relies on the incorporation of radioactive isotopes (e.g., ^{3}H, ^{14}C) into specific molecules. The radioactive particles expose a photographic film sensitive to the radiation placed on the sample. The radioactive areas appear as black dots on the film.
- ✓ **Applications**: Study of protein and nucleic acid synthesis. Monitoring of intracellular transport of certain molecules. Analysis of cellular metabolism.
- ✓ **Examples of isotopes used**: ^{3}H (tritium), ^{14}C (carbon-14), ^{32}P (phosphorus-32).

5. Contribution of each electronic contrast method to the morphological analysis of the sample

Tableau 4 : Summary of the morphological contributions of contrast processes

Procédé	Morphological Contribution	Typical Observation
Positive Staning	Visualizing internal structures with precision	Cell organelles, membranes
Negative Staining	Fine details of small and isolated structures	Viruses, ribosomes, macromolecules
Metallic Shading	Three-dimensional relief and surface topography	DNA, fibrous proteins, viruses
Autoradiography	Specific localization of labeled molecules	Protein synthesis, labeled DNA

In summary: The various contrast methods in electron microscopy allow for the visualization of diverse structures with high precision.

- ✓ **Positive Staning** : Internal structures.
- ✓ **Negative Staning** : Isolated particles (virus, ribosomes).
- ✓ **Metallic shading**: Relief and surface details.
- ✓ **Autoradiography** : Monitoring radioactive molecules within cells.

8. Operating principle of the Scanning Electron Microscope (SEM).

The **S**canning **E**lectron **M**icroscope (**SEM**) is a tool used to examine the surface of samples with very high resolution. Unlike the Transmission Electron Microscope (TEM), which observes the interior of ultrathin samples, the SEM allows for three-dimensional (3D) visualization of surface structures.

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1. Principe Fondamental :

- ✓ **An electron beam** is generated by an electron gun and focused by electromagnetic lenses. This beam scans the sample surface point by point along a raster scan path (line by line).
- ✓ **Primary electrons** interact with atoms on the sample surface, producing various secondary signals:
- ✓ **Secondary electrons**: Produced near the surface, they provide information about the surface morphology.
- ✓ **Backscattered electrons**: Reflected by heavy atoms, they provide chemical contrast.
- ✓ These signals are captured by specific detectors, then transformed into a digital image displayed on a screen.

2. Field of SEM: Analysis of fractures and structural defects. Characterization of nanomaterials.

- ✓ **Biology and Medicine**: Observation of cells, biological tissues, and bacterial biofilms. Study of host-pathogen interactions. Analysis of cellular and viral morphologies.
- ✓ **Microbiology**: Observation of microorganisms (bacteria, viruses, fungi). Study of microbial external structures (flagellates, cilia).
- ✓ **Materials Science**: Study of the structure of metals, alloys, and composites.
- ✓ **Geology and Mineralogy**: Analysis of rocks, minerals, and fossils. Study of mineral inclusions. Identification of crystalline structures.

3. Steps in preparing a sample for SEM using the cryo-etching technique

a) Fixation: Preserves biological structures in their natural state. **Method**: Uses chemical fixatives (e.g., glutaraldehyde, formaldehyde).

b) Rapid freezing (Cryofixation): Preserves the sample's native state. **Method**: Ultra-rapid freezing using liquid nitrogen (-196 °C) or a cryogenic mixture. Prevents the formation of ice crystals, which could damage cellular structures.

c) Freeze-fracture: Mechanically breaks the frozen sample to expose internal surfaces. **Method**: The sample is fractured under vacuum at very low temperatures (-100 °C to -150 °C). The fracture often follows membrane planes, revealing unique internal structures.

d) Metallic shading: Creates a three-dimensional relief on the sample. **Method**: Deposition of a thin layer of heavy metal (gold, platinum) at an oblique angle. This improves contrast and allows for better detection of details.

e) Controlled sublimation: The objective is to gently remove the remaining thin layer of ice to expose the fracture details. **Method**: Gentle heating under high vacuum to allow the ice to sublimate without altering the structure.

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f) SEM observation: The sample is placed in the SEM analysis chamber. An electron beam scans the surface to capture the emitted signals (secondary electrons, backscattered electrons). The data is converted into a high-resolution three-dimensional image.

g) Applications of SEM Cryo-Etching:

- ✓ Study of cell and subcellular membranes.
- ✓ Observation of macromolecular complexes.
- ✓ Analysis of interactions between cellular organelles.
- ✓ Visualization of the internal structures of bacterial biofilms.

9. Methods for isolating cellular components (DUC, DGU)

The isolation of cellular components aims to separate and purify cellular organelles and macromolecules in order to analyze them individually. Two main methods are used:

- ✓ **DUC** : **Differential UltraCentrifugation**,
- ✓ **DGU** : **Density Gradient Ultracentrifugation**.

Both techniques exploit the differences in size, mass, and density of cellular components to separate them efficiently.

1. Structures obtained in each pellet after application d'DUC.

Principle: this technique is based on the differential sedimentation rate of cellular components according to their **size** and **density** under the effect of a **high centrifugal force**.

Tableau 5 : Summary of the structures obtained at each stage of DUC :

Centrifugation Stage	Centrifugal force (g)	Duration	Sedimented structures (pile)
First stap (low speed)	1 000 - 2 000 g	10 min	Nuclei, cell debris, whole cells
2nd step (intermediate speed)	10 000 - 15 000 g	20 min	Mitochondries, lysosomes, peroxysomes
3th step (high speed)	100 000 g	1 H	Ribosomes, fragments of the RER, small membranous vesicles
4th step (very high speed)	300 000 g	2 H	Ribonucleoprotein complexes, viruses, soluble macromolecules

2. Structures obtained in each pellet after DGU application:

Principle: Density gradient ultracentrifugation (DGU) separates cellular components based on their flotation density in a continuous or discontinuous gradient (often based on sucrose or cesium chloride). Each component migrates until it reaches a zone where its density equals that of the gradient.

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Continuous (Progressive) Gradient: The density increases progressively from the top to the bottom of the centrifuge tube. The cellular components migrate until they reach **an isopycnic equilibrium zone**, where their density matches that of the gradient.

Tableau 6 : Location of structures according to density:

Approximate Density (g/mL)	Separated cellular structures
1,05 - 1,10	Membranes plasmiques, vésicules légères
1,12 - 1,18	Appareil de Golgi, REL
1,18 - 1,25	Mitochondries, lysosomes, peroxysomes
1,30 - 1,35	Ribosomes, fragments du REG
1,35 - 1,40	Nucléoprotéines, complexes ribonucléoprotéiques
>1,40	Virus (selon leur densité), macromolécules très denses

In summary :

- ✓ **DUC:** is fast and efficient for coarse separation based on the size and mass of cellular components.
- ✓ **DGU**: allows for more precise separation, based on equilibrium density, suitable for detailed studies of cellular components.

These two techniques are complementary and often used together for better characterization of cellular structures.

10. Contrast and analysis techniques applicable to isolated cellular structures

After the isolation of cellular components (by differential ultracentrifugation or density gradient centrifugation), several techniques are used to analyze and characterize them. These methods include microscopic (**negative contrast**) and biochemical **(chromatography, electrophoresis**) approaches.

1. Negative Contrast (Electron Microscopy): This technique is primarily used in transmission electron microscopy (TEM), the principle of which has already been explained. Its value lies in the study of the fine morphology of viruses, ribosomes, vesicles, and isolated macromolecules, as well as the analysis of viral particles and macromolecular complexes.

2. Chromatography (Biochemical Analysis): This is a technique for separating cellular components based on their physical or chemical properties:

- ✓ Column chromatography (ion-exchange, molecular exclusion, affinity).
- ✓ **H**igh-**P**erformance **L**iquid **C**hromatography (**HPLC**).

Each molecule migrates at a different rate depending on its affinity for the stationary and mobile phases.

- ➢ **Applications**:
  - ✓ Separation and purification of proteins, enzymes, lipids, and nucleic acids.
  - ✓ Study of the biochemical properties of isolated organelles and complexes.

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3. Electrophoresis (Biochemical Analysis): A technique based on the migration of charged molecules in an electric field. Molecules are separated according to their size, electrical charge, and sometimes their three-dimensional structure.

- **Common Techniques**:
  - ✓ SDS-PAGE (Sodium-Polyacrylamide Gel Electrophoresis) for proteins.
  - ✓ Agarose Gel Electrophoresis (SDS-PAGE) for nucleic acids.
- **Applications**:
  - ✓ Analysis of isolated proteins and nucleic acids.
  - ✓ Determination of molecular size.
  - ✓ Study of the biochemical and structural properties of macromolecules.

In summary: These tools are essential in cell and molecular biology for accurately characterizing isolated cellular components.

- ❖ **Negative contrast**: Morphological study of structures isolated by TEM.
- ❖ **Chromatography**: Separation and biochemical purification of components.
- ❖ **Electrophoresis**: Analysis of the biochemical properties of proteins and nucleic acids.

Tableau 7 : Comparison of Techniques

Technique	Type d'analyse	Main targets	Advantages	Disadvantages
Negative contrast	Microscopic	Virus, ribosomes, vésicules	High resolution	Complex preparation
Chromatography	Biochemical	Proteins, lipides, enzymes	Precise separation	Expensive equipment
Électrophoresis	Biochemical	Protéines, acides nucléiques	Qualitative and quantitative analysis	Possible denaturation

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