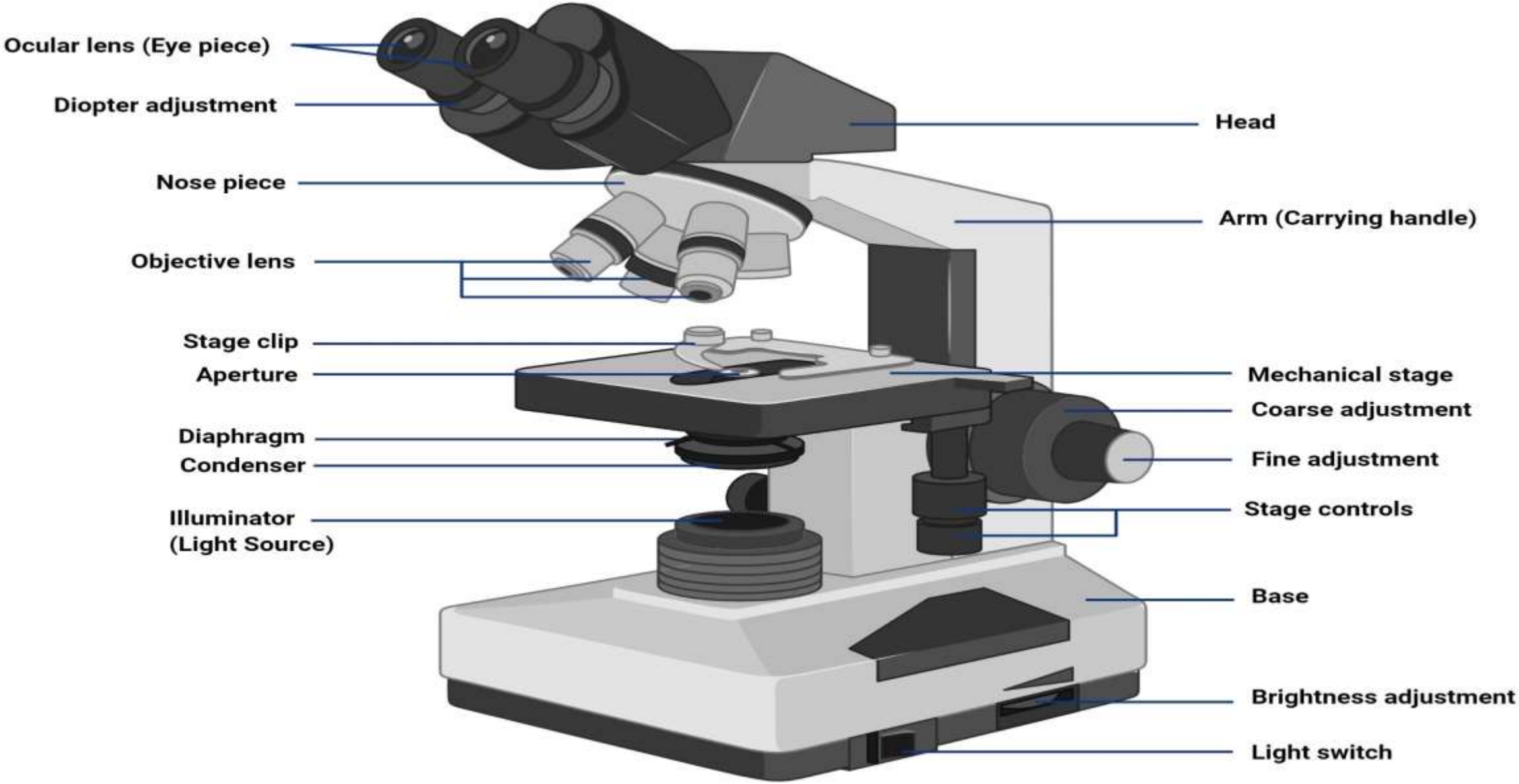
# GENERAL MICROBIOLOGY

# UNIT II

# Brightfield Microscope

- **Brightfield Microscope is also known as the Compound Light Microscope.**
- This microscope is used to view fixed and live specimens, that have been stained with basic stains which gives a contrast between the image and the image background. It is specially designed with magnifying glasses known as lenses that modify the specimen to produce an image seen through the eyepiece.
- The specimens used are prepared initially by staining to introduce color for easy contracting characterization. The colored specimens will have a refractive index that will differentiate it from the surrounding, presenting a combination of absorption and refractive contrast.
- The functioning of the microscope is based on its ability to produce a high-resolution image from an adequately provided light source, focused on the image, producing a high-quality image.
- The specimen which is placed on a microscopic slide is viewed under oil immersion or/and covered with a coverslip.


Ocular lens (Eye piece)
Diopter adjustment
Nose piece
Objective lens
Stage clip
Aperture
Diaphragm
Condenser
Illuminator
(Light Source)
Head
Arm (Carrying handle)
Mechanical stage
Coarse adjustment
Fine adjustment
Stage controls
Base
Brightness adjustment
Light switch

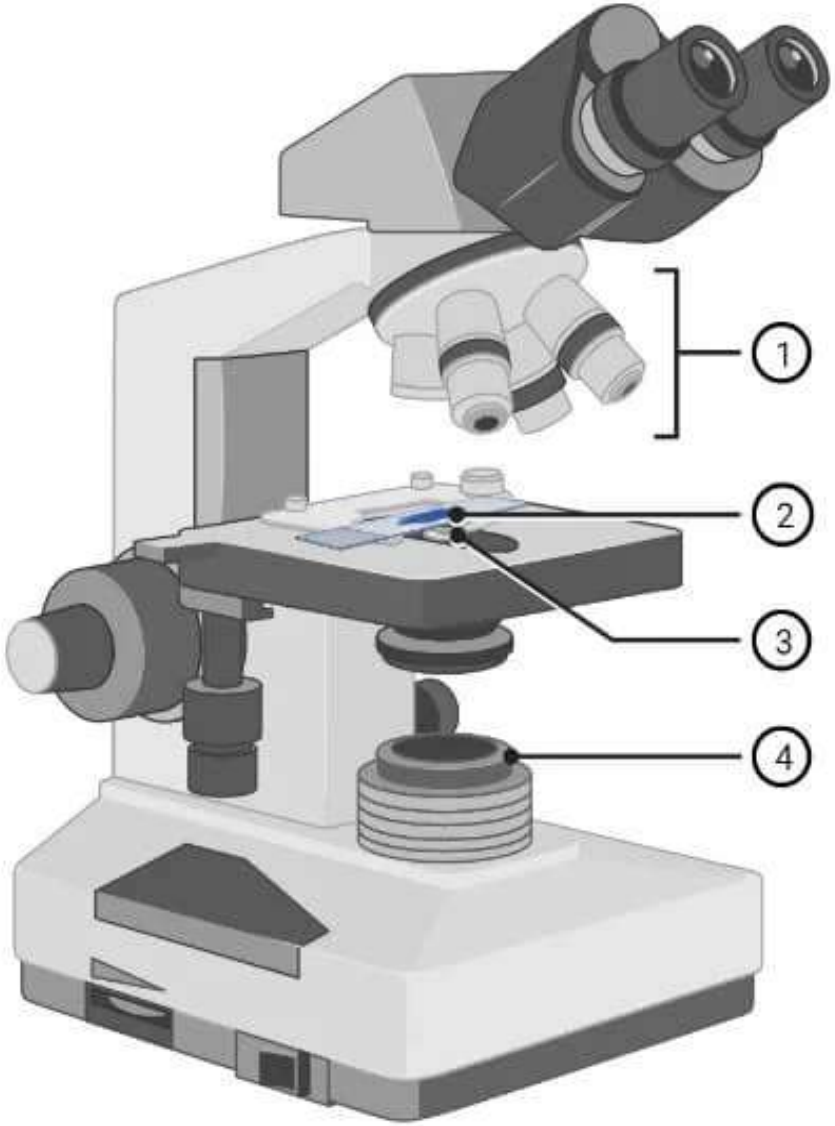
- The brightfield microscope is made up of various parts, including
- **Eyepiece (Ocular lens)** – it has two eyepiece lenses at the top of the microscope which focuses the image from the objective lenses. this is where you see the formed image from, with your eyes.
- **The objective lenses** which are made up of six or more glass lenses, which make a clear image clear from the specimen or the object that is being focused.
- **Two focusing knobs** The fine adjustment knob and the coarse adjustment knob, found on the microscopes' arm, which can move the stage or the nosepiece to focus on the image. Their function is to ensure the production of a sharp image with clarity.
- **The stage** is found just below the objectives and this is where the specimen is placed, allowing movement of the specimen around for better viewing with the flexible knobs and it is where the light is focused on.
- **The condenser**: It is mounted below the stage which focuses a beam of light onto the specimen. It can be fixed or movable, to adjust the quality of light, but this entirely depends on the microscope.
- **The arm**: This is a sturdy metallic backbone of the microscope, used to carry and move the microscope from one place to another. They also hold the microscope **base** which is the stand of the microscope. The arm and the base hold all the microscopic parts.
- It has a **light illuminator** or a **mirror** found at the base or on the microscope's nosepiece.
- The **nosepiece** has about two to five objective lenses with different magnifying power. It can move round to any position depending on the objective lens to focus on the image.
- **An aperture diaphragm (contrast)**: It controls the diameter of the beam of light that passes through the condenser. When the condenser is almost closed, the light comes through to the center of the condenser creating high contrast and when the condenser is widely open, the image is very bright with very low contrast.

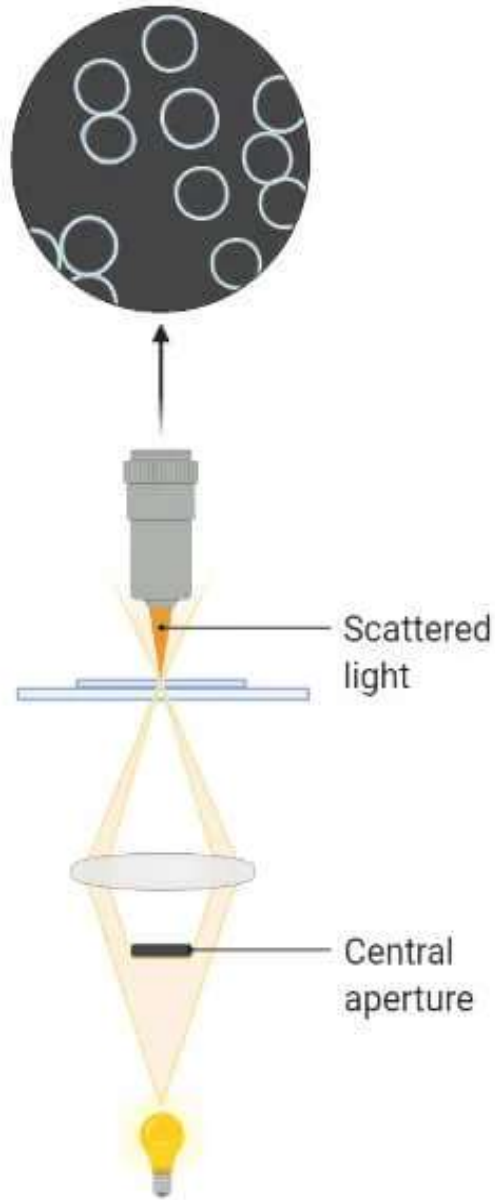
# Dark field microscope

- Microbiology, the branch of science that has so vastly extended and expanded our knowledge of the living world, owes its existence to Antoni van Leeuwenhoek.
- In 1673, with the aid of a crude microscope consisting of a biconcave lens enclosed in two metal plates, Leeuwenhoek introduced the world to the existence of microbial forms of life.
- Over the years, microscopes have evolved from the simple, single-lens instrument of Leeuwenhoek, with a magnification of 300 X, to the present-day electron microscopes capable of magnifications greater than 250,000X.
- Microscopes are designated as either light microscopes or electron microscopes.
- Light microscopes use visible light or ultraviolet rays to illuminate specimens. They include **brightfield**, darkfield, phase-contrast, and fluorescent instruments.
- This is similar to the ordinary light microscope; however, the condenser system is modified so that the specimen is not illuminated directly.
- The condenser directs the light obliquely so that the light is deflected or scattered from the specimen, which then appears bright against a dark background.
- Living specimens may be observed more readily with darkfield than with brightfield microscopy.

- A dark field microscope is arranged so that the light source is blocked off, causing light to scatter as it hits the specimen.
- This is ideal for making objects with refractive values similar to the background appear bright against a dark background.
- When light hits an object, rays are scattered in all azimuths or directions. The design of the dark field microscope is such that it removes the dispersed light, or zeroth order, so that only the scattered beams hit the sample.
- The introduction of a condenser and/or stop below the stage ensures that these light rays will hit the specimen at different angles, rather than as a direct light source above/below the object.
- The result is a “cone of light” where rays are diffracted, reflected and/or refracted off the object, ultimately, allowing the individual to view a specimen in dark field.
- Dark-field microscopy is a very simple yet effective technique.
- It is well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual, water-borne, single-celled organisms.
- Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.
- Dark-field microscopy techniques are almost entirely free of artifacts, due to the nature of the process.

# Darkfield Microscope



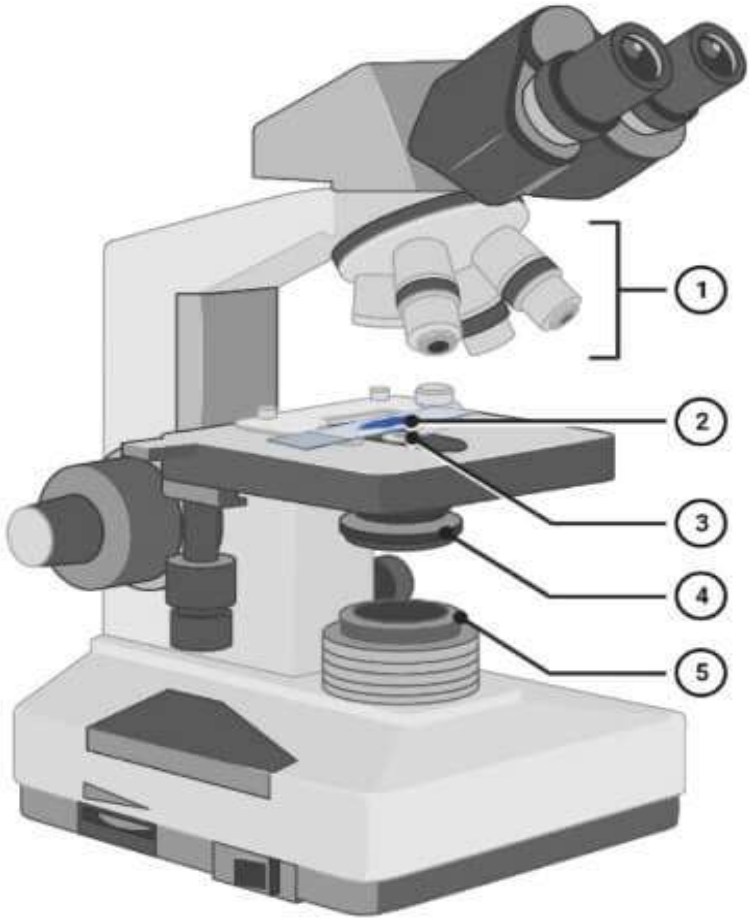



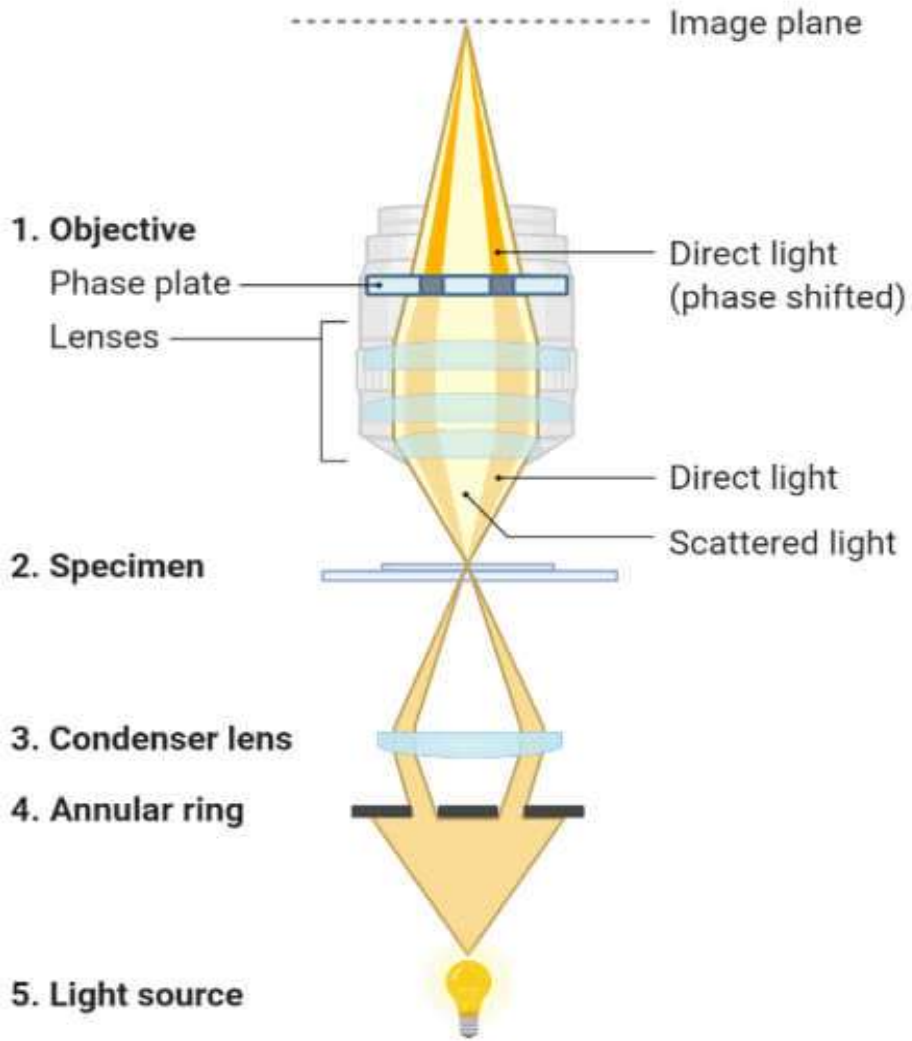

1. Objective
2. Specimen
3. Condenser lens
4. Light source

# Phase Contrast Microscopy

- Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled condenser annulus) positioned in the substage condenser front focal plane.
- Wavefronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen.
- Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a phase plate and focused at the intermediate image plane to form the final phase-contrast image observed in the eyepieces.
- The annular diaphragm
- It is situated below the condenser.
- It is made up of a circular disc having a circular annular groove.
- The light rays are allowed to pass through the annular groove.
- Through the annular groove of the annular diaphragm, the light rays fall on the specimen or object to be studied.
- At the back focal plane of the objective develops an image.
- The annular phase plate is placed at this back focal plane.

# Phase Contrast Microscopy



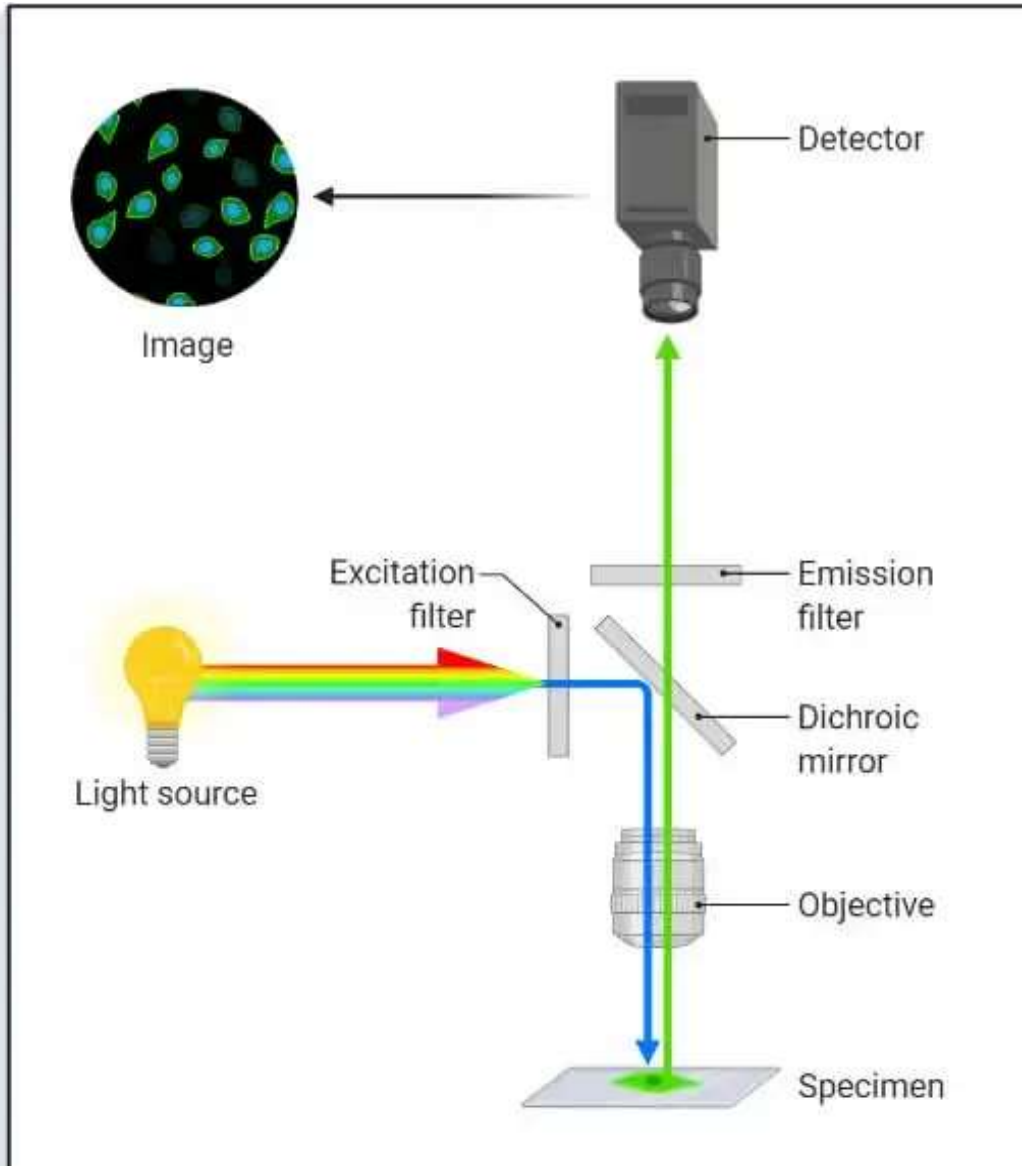
## The phase plate

- It is either a negative phase plate having a thick circular area or a positive phase plate having a thin circular groove. This thick or thin area in the phase plate is called the conjugate area.
- The phase plate is a transparent disc With the help of the annular diaphragm and the phase plate, the phase contrast is obtained in this microscope.
- This is obtained by separating the direct rays from the diffracted rays.
- The direct light rays pass through the annular groove whereas the diffracted light rays pass through the region outside the groove. Depending upon the different refractive indices of different cell components, the object to be studied shows a different degree of contrast in this microscope.
- Living cells can be observed in their natural state without previous fixation or labeling. It makes a highly transparent object more visible.
- No special preparation of fixation or staining etc. is needed to study an object under a phase-contrast microscope which saves a lot of time. Examining intracellular components of living cells at relatively high resolution. eg: The dynamic motility of **mitochondria**, mitotic chromosomes & vacuoles.
- It made it possible for biologists to study living cells and how they proliferate through cell division.
- Phase-contrast optical components can be added to virtually any brightfield microscope, provided the specialized phase objectives conform to the tube length parameters, and the condenser will accept an annular phase ring of the correct size.

# Fluorescence Microscope

- **A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study the properties of organic or inorganic substances.**
- Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation while phosphorescence is a specific type of photoluminescence related to fluorescence.
- The initial step in the observation of the sample through a fluorescence microscope includes labelling the sample with fluorescent dyes. Then, light source which emits white light is allowed to fall onto the excitation filter This filter selects the light of a specific wavelength that can excite the fluorescent molecules tagged in the specimen and this excitation light incidents onto the dichroic mirror. The light after reflection from the dichroic mirror passes onto the specimen after emerging from the objective lens. This small wavelength light falls into the specimen stained with a fluorescent dye that results in emission of high wavelength light which passes again through the condenser lens and dichroic mirror. This allows green light in maximum along with some blue light to pass towards the emission filter. However, this filter only permits the longer wavelength green light to pass into the eyepiece and detector while at the same time rejecting the blue light completely. The detector detects the green light and permits it to fall back onto the specimen thereby, forming fluorescent green specimens against a dark background.

# Fluorescence Microscopy



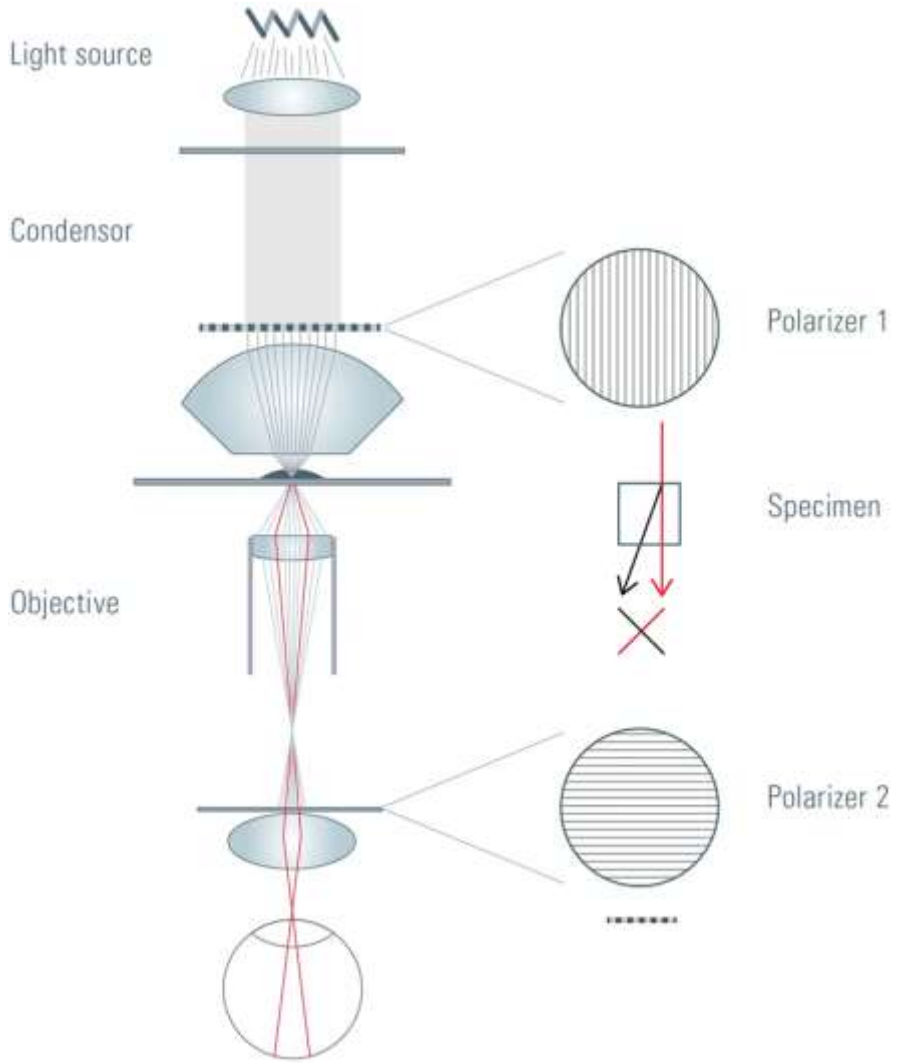
Some of the components of fluorescence microscope are as follows:

- **Fluorophore:** These are reactive fluorescent dyes which form a fluorescent image by generating highly contrasted visible green light after being activated by highly illuminating UV radiation.
- **Light Source:** Major light sources include xenon arc lamps, mercury-vapour lamps, lasers and high-power LEDs. A simple epifluorescent microscope makes use of a light source made up of xenon lamps, mercury lamps, and LEDs. On the other hand, laser light is mostly used by the advanced confocal fluorescence microscope.
- **Excitation filter:** The excitation filter is a bandpass filter that functions to narrow the wavelength of the light that illuminates the sample by blocking other sources of exciting light. Such light of shorter wavelength can be easily absorbed by the fluorescent dye.
- **Dichroic Mirror:** It is also known as dichromatic mirror or beam-splitter. It selectively reflects or transmits light with specific wavelengths.
- **Objective/ Condenser lens:** The light is then directed through a system of optics that serves as both a condenser, gathering the light into a narrow beam on the sample, and a focusing objective for the light emitted back by the specimen.
- **Sample stage:** The sample stage holds the specimen and contains x, y, and z-axis movements that can be controlled manually or by a computer.
- **Emission filter:** It is a bandpass filter that functions similarly to an excitation filter. The light emitted from the specimen includes light reflected from the sample, which will be the excitation wavelength as well as light emitted from the fluorescent components of the specimen. Since, certain samples may include different fluorescent material, light of different wavelengths may be emitted. It allows fluorophore light radiations to pass while blocking excitation light.
- **Eyepieces and detector:** The light after passing through an emission filter is directed toward a set of eyepieces to facilitate the user to see or to a camera. The fluorescence images can then be quantitatively analyzed using a combination of digital imaging and image processing.

# Polarization microscope

- A polarizing microscope is a type of microscope that uses polarized light to view specimens.
- Polarized light is light that vibrates in a specific plane, rather than vibrating randomly in all directions like normal light. When light passes through certain types of materials, it can become polarized. A polarizing microscope uses this property of light to reveal the internal structure and properties of a specimen.
- It is commonly used to observe minerals, crystals, and other transparent or semi-transparent materials, as well as to analyze the structure and properties of these materials.
- In a polarized light microscope, the light source and sample are separated by a polarizer. Before reaching the sample, the polarized light source is transformed to plane-polarized light. This polarized light strikes a material, which forms two wave components at right angles to one another. These two waves are known as common and exceptional light beams. The specimen is traversed by the waves in several phases. An analyzer then combines them using constructive and destructive interference. This results in the final creation of an image with strong contrast.
- The polarizer is positioned beneath the specimen stage and may be turned 360 degrees. It aids in polarizing the light falling on the specimen.
- Polarizing filters are the most necessary component of a polarized light microscope. Typically, polarizing filters consist of two components: the polarizer and the analyzer

- The analyzer is positioned above the objective and is sometimes rotatable. It mixes the many rays emitted by the specimen to get the final image.
- Polarizing microscopes are also used in biology to study the structure and properties of biological specimens, such as cells, tissues, and organelles. They can be used to analyze the organization and orientation of molecules within cells and to study the effects of different treatments on biological samples.
- Polarizing microscopes are used in geology to study the structure and properties of rocks and minerals. They can be used to identify minerals, analyze the composition of rocks, and study the effects of different geological processes on the materials.
- Polarizing microscopes are often used study of muscle tissues, visualize the arrangement and orientation of collagen fibers in tissues, cellulose in plant cell walls, nerve fibers, starch grain in plants, spindle apparatus in cell division



# Electron Microscope

- An electron microscope is a microscope that uses a beam of accelerated electrons as a source of illumination. It is a special type of microscope having a high resolution of images, able to magnify objects in nanometers, which are formed by controlled use of electrons in a vacuum captured on a phosphorescent screen.
- Ernst Ruska (1906-1988), a German engineer and academic professor, built the first Electron Microscope in 1931, and the same principles behind his prototype still govern modern EMs.
- The electron gun generates electrons. Two sets of condenser lenses focus the electron beam on the specimen and then into a thin tight beam. To move electrons down the column, an accelerating voltage (mostly between 100 kV-1000 kV) is applied between the tungsten filament and anode. The specimen to be examined is made extremely thin, at least 200 times thinner than those used in the optical microscope. Ultra-thin sections of 20-100 nm are cut which is already placed on the specimen holder.
- The electronic beam passes through the specimen and electrons are scattered depending upon the thickness or refractive index of different parts of the specimen. The denser regions in the specimen scatter more electrons and therefore appear darker in the image since fewer electrons strike that area of the screen. In contrast, transparent regions are brighter. The electron beam coming out of the specimen passes to the objective lens, which has high power and forms the intermediate magnified image.

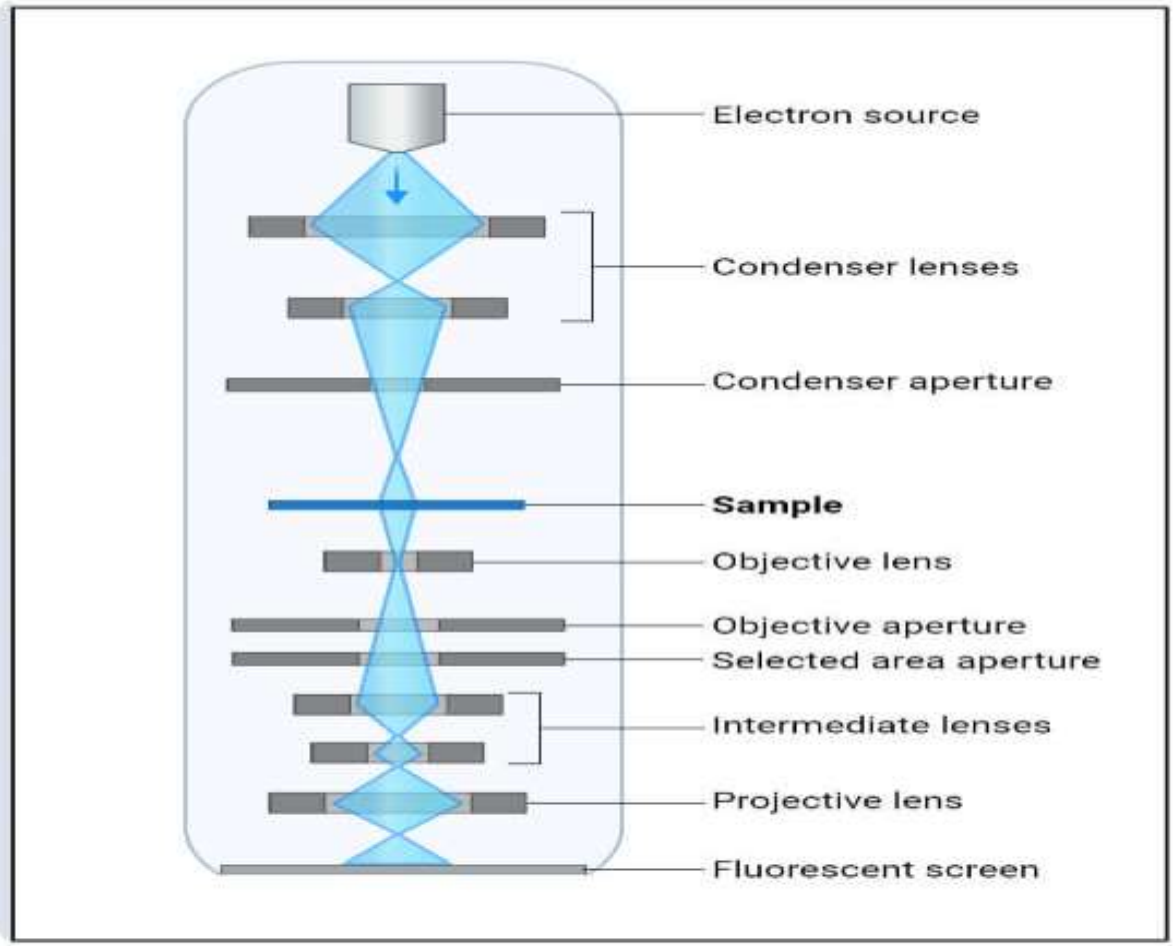
## Types of Electron microscope

- There are two types of electron microscopes, with different operating styles:

### 1. Transmission Electron Microscope (TEM)

- The transmission electron microscope is used to view thin specimens through which electrons can pass generating a projection image.
- The TEM is analogous in many ways to the conventional (compound) light microscope.
- TEM is used, among other things, to image the interior of cells (in thin sections), the structure of protein molecules (contrasted by metal shadowing), the organization of molecules in viruses and cytoskeletal filaments (prepared by the negative staining technique), and the arrangement of protein molecules in cell membranes (by freeze-fracture).

# Transmission Electron Microscopy (TEM)



# 2. Scanning Electron Microscope (SEM)

- Conventional scanning electron microscopy depends on the emission of secondary electrons from the surface of a specimen. Because of its great depth of focus, a scanning electron microscope is the EM analog of a stereo light microscope.
- It provides detailed images of the surfaces of cells and whole organisms that are not possible by TEM. It can also be used for particle counting and size determination, and for process control. It is termed a scanning electron microscope because the image is formed by scanning a focused electron beam onto the surface of the specimen in a raster pattern.

**Parts of Electron Microscope**

- Electron Microscope is in the form of a tall vacuum column that is vertically mounted. It has the following components:

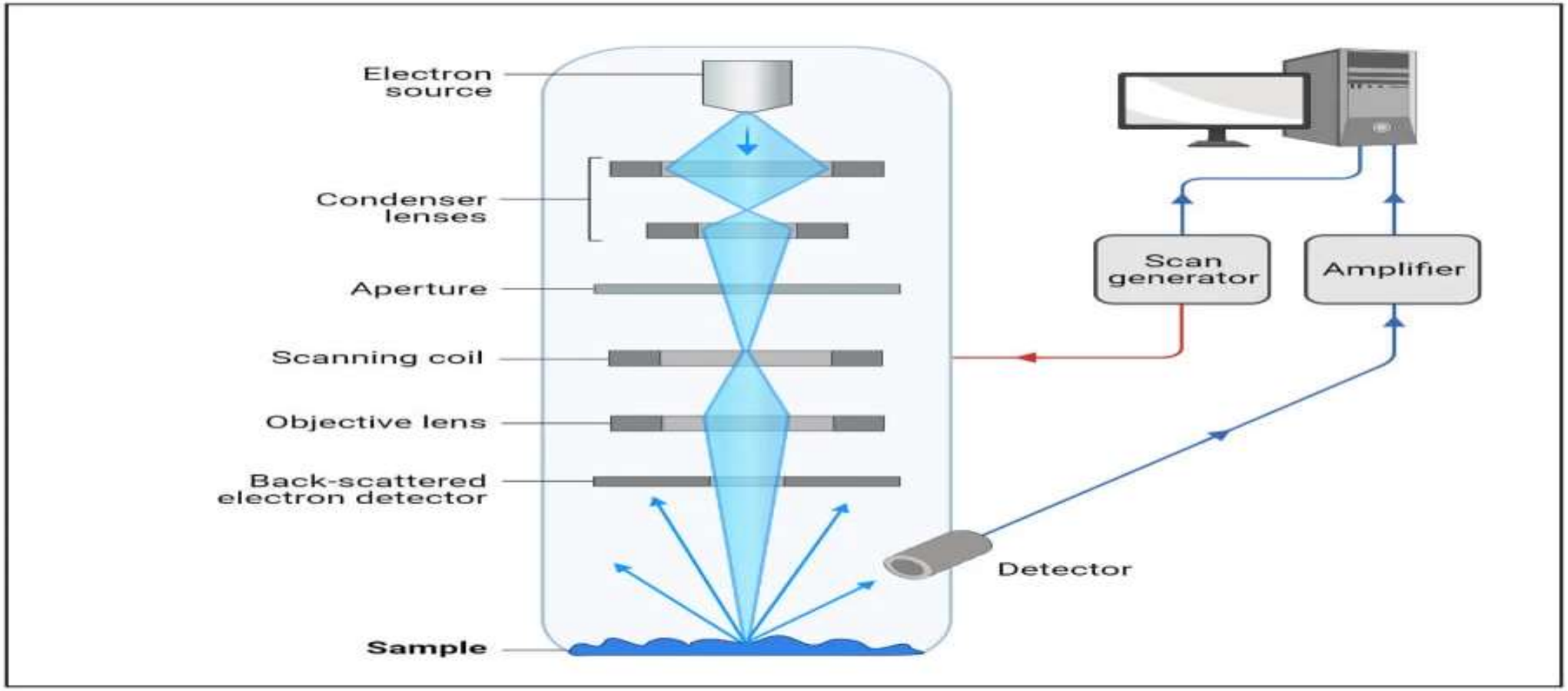
**1. Electron gun**

- The electron gun is a heated tungsten filament, which generates electrons.

**2. Electromagnetic lenses**

- **The condenser lens** focuses the electron beam on the specimen. A second condenser lens forms the electrons into a thin tight beam. The electron beam coming out of the specimen passes down the second of magnetic coils called the **objective lens**, which has high power and forms the intermediate magnified image.
- The third set of magnetic lenses called **projector (ocular) lenses** produce the final further magnified image. Each of these lenses acts as an image magnifier all the while maintaining an incredible level of detail and resolution.

# Scanning Electron Microscopy (SEM)



### 3. Specimen Holder

- The specimen holder is an extremely thin film of carbon or collodion held by a metal grid.

### 4. Image viewing and Recording System

- The final image is projected on a fluorescent screen.
- Below the fluorescent screen is a camera for recording the image.

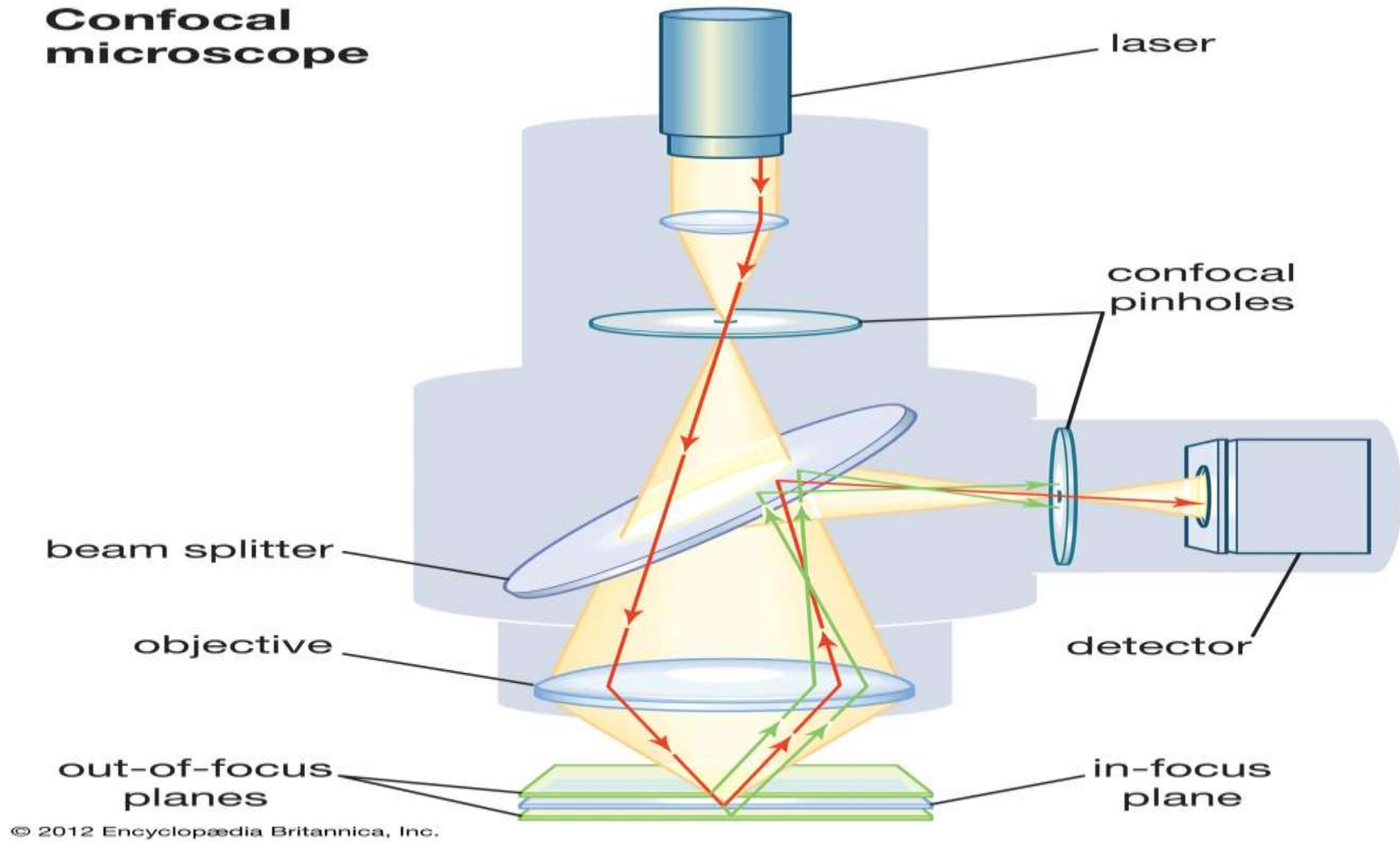
### Applications of Electron microscope

- Electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals.
- Industrially, electron microscopes are often used for quality control and failure analysis.
- Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the images.
- The science of microbiology owes its development to the electron microscope. The study of microorganisms like bacteria, virus, and other pathogens have made the treatment of diseases very effective.

# CONFOCAL MICROSCOPE

- A confocal microscope is a specialized type of optical microscope that provides high-resolution images of specimens by using a focused laser beam and pinhole apertures.
- A confocal microscope is an advanced optical imaging device that uses laser scanning and a pinhole aperture to capture high-resolution, three-dimensional images of specimens. By focusing on a single plane and eliminating out-of-focus light, it provides detailed and clear visualization of fine structures within biological tissues or materials, making it a valuable tool for precise and in-depth analysis in scientific research.
- **PRINCIPLE :**
- A confocal microscope enhances image clarity and resolution by using a point illumination system and spatial filtering to eliminate out-of-focus light. Here's how it works:
- **Point Illumination**: Unlike wide-field microscopes that illuminate the entire specimen, a confocal microscope uses a focused laser beam to illuminate a very small, specific area of the specimen at a time. This illumination is typically between 0.25 and 0.8 microns in diameter.
- **Optical Sectioning**: The microscope captures light from a thin, defined optical plane within the specimen. A pinhole aperture positioned in front of the detector blocks out-of-focus light from above and below the focal plane, ensuring that only light from the precise focal point is detected.
- **Beam Scanning**: To create an image, the focused beam is scanned across the specimen using two oscillating mirrors that move the beam along the X and Y axes. This scanning can be done in a raster pattern, with rapid flyback movements returning the beam to the start of the scan without collecting data.

# Confocal microscope



- **Data Collection**: The emitted fluorescence from the specimen is collected through the pinhole, and the detector captures the light from the focused area. The collected data is processed to produce high-resolution, contrast-rich images of optical sections.
- **3D Imaging**: By scanning multiple optical planes (z-stack) and compiling the data, the confocal microscope can create detailed three-dimensional images of the specimen. Modern systems with multiple lasers and filters allow for multicolor imaging and more complex analysis.

## APPLICATIONS :

- The Confocal Microscope is used in a wide range of fields including Biomedical sciences, Cells Biology, genetics, Microbiology, Developmental Biology, Spectroscopy, Nanoscience (nanoimaging), and Quantum Optics.
- In Biomedical sciences, it is used in the analysis of eye corneal infections, by quantifying and qualitatively analyzing the endothelial cells of the cornea.
- Used to identify the presence of fungal elements in the corneal stroma, during keratomycosis infection, or rapid diagnosis and quick therapeutic response.
- It is used in pharmaceutical industries, to ensure the maintenance of thin-film pharmaceuticals, allowing control of the quality and uniformity of drug distribution.
- It is used to retrieve data from some 3D optical storage systems. This has helped in quantifying the age of Magdalen’s papyrus.

# Sterilization and Its Methods

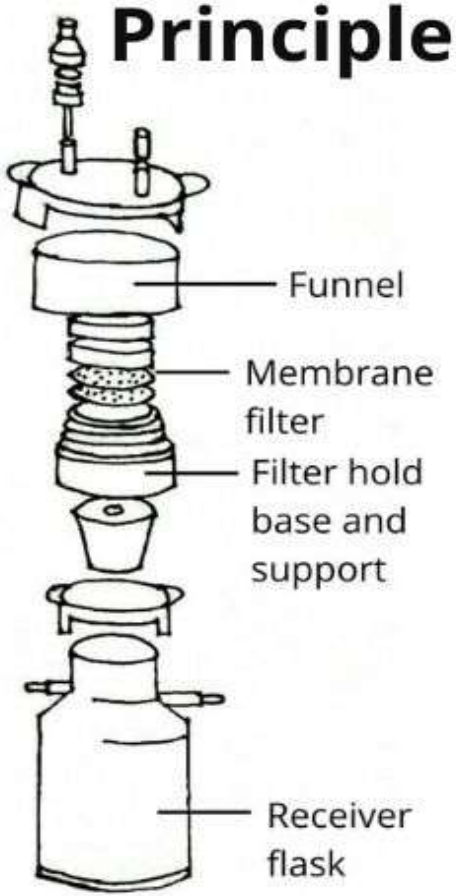
- **Sterilization** is the process of removing or destroying all forms of microbial life, including bacteria, viruses, fungi, and spores, from an object or surface. It is crucial in various fields like medicine, food processing, pharmaceuticals, and laboratory research to prevent contamination and ensure safety.
- Here are some common **methods of sterilization**:

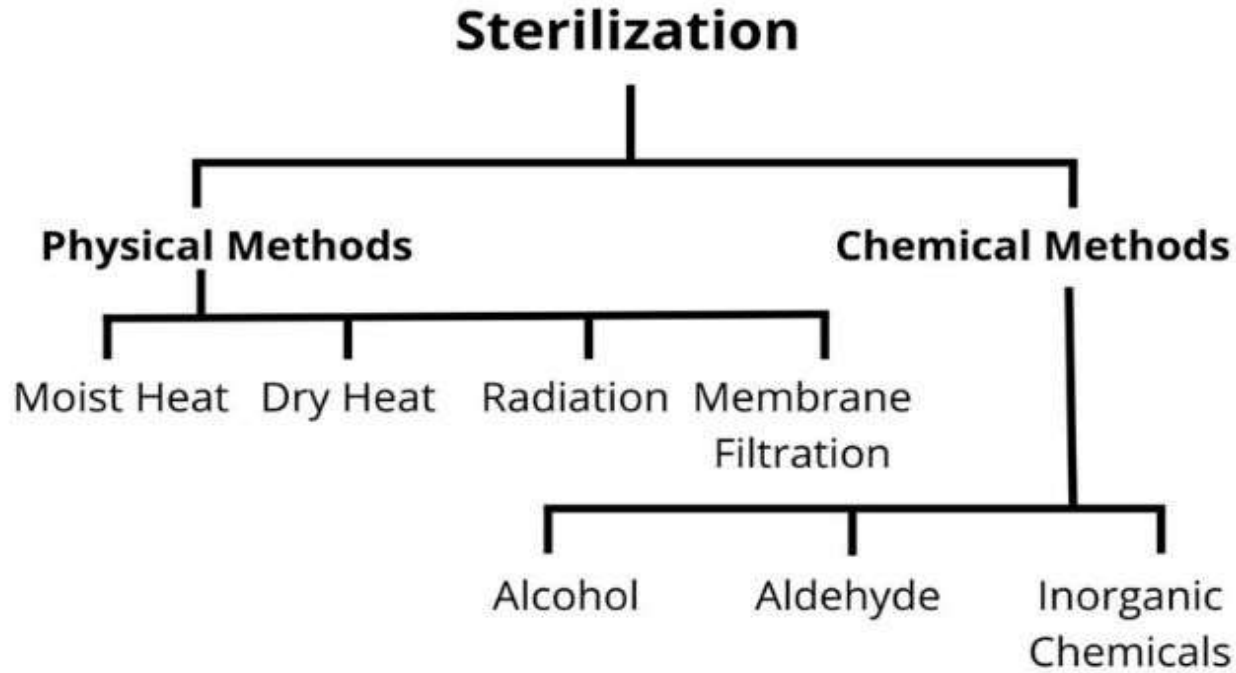
**1. Physical Methods**

- **Heat Sterilization**:
  - **Moist Heat (Autoclaving)**: Uses steam at high pressure and temperature (typically 121°C for 15-20 minutes). It is one of the most effective sterilization methods for equipment, surgical instruments, and liquids.
  - **Dry Heat**: Involves hot air (160-180°C) for longer periods, typically 1-2 hours. It is used for sterilizing glassware, metal instruments, and powders, but it is slower and less efficient than moist heat.
- **Filtration**:
  - This method is used for sterilizing heat-sensitive liquids (such as vaccines, antibiotics) or gases by passing them through a filter with pores small enough to trap microorganisms.
- **Radiation Sterilization**:
  - **Ultraviolet (UV) Radiation**: UV light is used to sterilize surfaces, air, and water by damaging microbial DNA, thus preventing reproduction. It is commonly used in laboratories, hospitals, and water purification.
  - **Gamma Radiation**: High-energy gamma rays are used to sterilize medical devices, food products, and pharmaceuticals. It penetrates materials and kills microorganisms by disrupting their DNA.

# Sterilization Techniques

## Principle, Methods, Requirements, Procedures



## 2. Chemical Methods

- **Ethylene Oxide Gas (ETO)**: ETO is a widely used gas for sterilizing heat-sensitive materials, such as medical instruments, plastics, and electronics. It is highly effective but requires careful handling due to its toxicity and flammability.
- **Hydrogen Peroxide**: It can be used in liquid form or as vapor for sterilizing surfaces, medical instruments, and even air. It works by generating reactive oxygen species that kill microorganisms.
- **Glutaraldehyde**: A potent disinfectant and sterilizing agent used for equipment like endoscopes and surgical tools. It is effective at killing bacteria, viruses, and fungi.
- **Formaldehyde**: Often used in gas form to sterilize rooms or equipment. However, due to its toxicity and carcinogenic properties, its use has become less common in favor of safer alternatives.

## 3. Mechanical Methods

- **Pressure Sterilization (Autoclaving)**: Autoclaving uses both heat and pressure to sterilize materials, particularly in medical and laboratory settings. This method is highly effective in destroying both vegetative cells and spores.

## 4. Gas Sterilization

- **Ozone Sterilization**: Ozone ($O_3$) is a powerful oxidizing agent that can be used to sterilize water, air, and surfaces. Ozone can destroy bacteria and viruses through oxidation, which disrupts their cellular structures.

## 5. Cold Sterilization

- **Low-temperature Sterilization**: This refers to methods such as hydrogen peroxide vapor, ozone, and ethylene oxide, which can sterilize at lower temperatures, making them suitable for items that can't withstand heat.

## 6. Desiccation (Drying):

- This method involves drying out the microorganism by removing the water necessary for its survival. Some bacteria can survive desiccation, so this method is not always 100% reliable for complete sterilization.
- The appropriate sterilization method depends on the nature of the material being sterilized, the level of sterility required, and the potential risks of contamination. For example, autoclaving is widely used for surgical instruments, while ethylene oxide or hydrogen peroxide is used for heat-sensitive equipment. Sterilization is crucial in maintaining hygiene, safety, and preventing infections in medical, industrial, and food environments.

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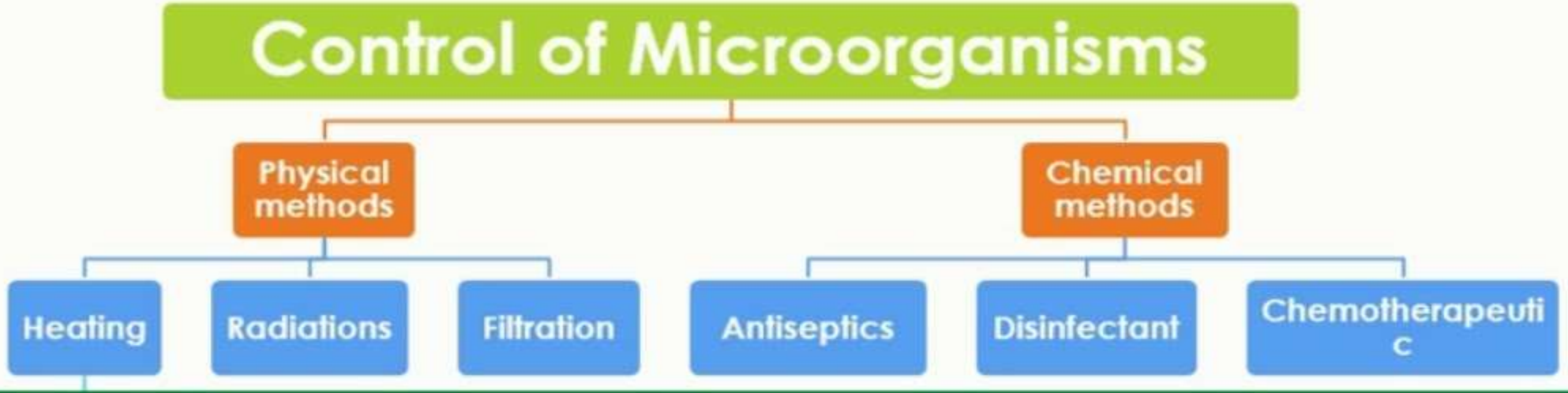


# Control of Microorganisms

- Dry heat can also be applied for relatively long periods of time (at least 2 hours) at temperatures up to 170 °C such as oven.
- Causes oxidation of chemical constituents of microbes and kills them.

- More effective because it penetrates cells better than dry heat does.
- Causes coagulation of protein and kill the microbes.
- Boiling and pasteurization uses moist heat.

# Isolation Of Microorganisms

- Isolation and preservation of microorganisms are key processes in microbiology to study, store, and maintain cultures for various applications, including research, clinical diagnostics, and industrial processes. Here's an overview of the isolation and preservation methods for different types of microorganisms:
- **1. Isolation of Microorganisms**
- The first step in studying microorganisms is isolating them from a mixed culture, usually obtained from an environmental or clinical sample.
- **A. Methods of Isolation:**

1. **Streak Plate Method:**
    1. **Purpose**: To separate individual colonies of microorganisms on a solid medium.
    2. **Procedure**: A sample is streaked across the surface of an agar plate in a pattern to spread the microorganisms. After incubation, individual colonies can be picked and sub-cultured.
2. **Pour Plate Method:**
    1. **Purpose**: To dilute the sample and allow the growth of discrete colonies.
    2. **Procedure**: A serial dilution of the sample is made, and the diluted sample is mixed with molten agar, poured into a petri dish, and allowed to solidify. Microorganisms grow within the agar or on the surface, where individual colonies can be identified.

**Spread Plate Method:**

•**Purpose**: To evenly distribute microorganisms on the surface of the agar for isolation.

•**Procedure**: A diluted sample is spread on the surface of an agar plate using a sterile spreader or loop.

**Selective and Differential Media:**

•**Purpose**: To isolate and differentiate specific groups of microorganisms based on their metabolic characteristics.

•**Examples**:

- •**Selective media**: MacConkey agar (selects for Gram-negative bacteria).
- •**Differential media**: Eosin methylene blue (EMB) agar (differentiates between lactose fermenters and non-fermenters).

**Serial Dilution Method:**

•**Purpose**: To dilute the microorganism sample in a series of steps, enabling the isolation of individual microorganisms.

•**Procedure**: A series of dilutions is prepared, and a small volume from each dilution is plated to obtain countable colonies.

**Immunological Methods:**

- **Purpose**: Used to isolate microorganisms based on specific antibodies or antigens.
- **Examples**: Using monoclonal antibodies or antigen-specific markers to separate a particular microorganism from a sample.

## 2. Preservation of Microorganisms

- Once microorganisms are isolated, it's essential to preserve them for future use in research or production.

### A. Methods of Preservation:

**1. Refrigeration (4°C):**

1. **Purpose**: Short-term storage of microorganisms.
2. **Method**: Cultures are stored at low temperatures to slow down the growth and metabolic activities of microorganisms, thus prolonging their viability for a short period (typically weeks to months).

**2. Freezing (−20°C to −80°C):**

1. **Purpose**: To store microorganisms for long periods.
2. **Method**: Cultures are stored in a freezer. Glycerol or another cryoprotectant is often added to prevent ice crystal formation, which can damage cells. Cultures can remain viable for years under these conditions.

**3. Lyophilization (Freeze-drying):**

1. **Purpose**: Long-term preservation of microorganisms in a dry state.
2. **Method**: The culture is first frozen and then dried under vacuum. This technique allows for long-term storage at room temperature while maintaining the viability of the microorganisms. It is commonly used for bacterial strains and fungi.

- **Cryopreservation:**
- **Purpose**: To store microorganisms in a state where they can be revived later.
- **Method**: Microorganisms are suspended in cryoprotectants (like glycerol or DMSO) and then stored at extremely low temperatures (−80°C or in liquid nitrogen, −196°C). This method is often used for preserving bacterial, fungal, and mammalian cell cultures.

**Subculturing:**

•**Purpose**: To maintain viable cultures over time.

•**Method**: Regular subculturing on fresh media ensures that microorganisms are kept in active growth and prevent the loss of viable cells.

**Agar Slants or Stabs:**

•**Purpose**: Long-term storage for bacteria.

•**Method**: Microorganisms are inoculated onto solid agar slants or stabs and stored at room temperature or in a refrigerator. This is a common method for bacterial cultures and fungi.

**Vitrification:**

•**Purpose**: To prevent ice crystal formation and ensure long-term survival.

•**Method**: A special cryoprotective technique where cells are cooled very rapidly, typically involving a glass-like solid state at ultra-low temperatures. This technique is used to preserve yeast, bacteria, and even viruses.

**Storage in Mineral Oil:**

•**Purpose**: Used for storing bacterial cultures.

•**Method**: Microorganisms are grown on agar slants and covered with mineral oil to prevent contamination and allow long-term storage.

## 3. Specific Considerations for Different Types of Microorganisms:

- **Bacteria:**
  - Bacteria are typically preserved by freezing, lyophilization, or by inoculating agar slants.
  - Special attention is given to preserving anaerobic bacteria by avoiding oxygen exposure during storage.
- **Fungi (Yeasts and Molds):**
  - Preservation is often done via lyophilization, freezing, or by storing them on agar slants.
  - Some yeasts are cryopreserved using glycerol as a cryoprotectant.
- **Viruses:**
  - Viruses are often preserved in liquid nitrogen at ultra-low temperatures.
  - They may also be stored in a lyophilized form for research purposes.
- **Algae:**
  - Preservation of algae typically involves keeping them at a constant temperature in liquid cultures or by drying in a controlled environment.
- **Protozoa:**
  - Protozoa are preserved by freezing, lyophilization, or in nutrient-rich media under controlled environmental conditions.

# Identification of Microorganisms

- **Identification of microorganisms using morphology** involves examining the physical characteristics of microorganisms, including their shape, size, structure, and arrangement. These characteristics can help classify and differentiate microorganisms such as bacteria, fungi, and algae, which are key for diagnostic purposes, research, and epidemiology.
- Here are the main **morphological features** used in identifying microorganisms:

**1. Shape**

- The shape of the microorganism is one of the primary features used for identification, especially in bacteria. Some common shapes include:
- **Cocci**: Spherical or round-shaped bacteria. Examples include:
  - *Streptococcus* (chains of cocci)
  - *Staphylococcus* (clusters of cocci)
- **Bacilli**: Rod-shaped bacteria. Examples include:
  - *Escherichia coli* (straight rods)
  - *Bacillus* (spore-forming rods)
- **Spirilla**: Spiral-shaped or helical bacteria. Examples include:
  - *Spirillum* (rigid spiral)
  - *Treponema* (flexible spiral)
- **Vibrio's**: Comma-shaped bacteria. Examples include:
  - *Vibrio cholerae* (the causative agent of cholera)
- **Filamentous**: Long, thread-like shapes that are often seen in fungi or actinomycetes.

## 2. Size

- The size of microorganisms can vary widely. Bacteria typically range from 0.5 microns to several microns in size. Larger microorganisms such as fungi and protozoa may range from 10 microns to hundreds of microns. Microscopic examination under a microscope is necessary to estimate size accurately.

## 3. Cell Wall Structure (Gram Staining)

- The structure of the microbial cell wall is crucial for identification. **Gram staining** is a technique that distinguishes bacteria into two main groups based on their cell wall properties:
- **Gram-positive bacteria**: These bacteria have a thick peptidoglycan layer in their cell wall that retains the crystal violet stain, turning them purple. Examples include:
  - *Staphylococcus aureus*
  - *Streptococcus pneumoniae*
- **Gram-negative bacteria**: These bacteria have a thinner peptidoglycan layer and an outer membrane, which doesn't retain the crystal violet but instead takes up the counterstain (safranin), making them appear pink. Examples include:
  - *Escherichia coli*
  - *Salmonella*

## 4. Arrangement

- The way microorganisms are arranged can be helpful in identification:
- **Cocci** may be arranged in clusters (e.g., *Staphylococcus*), chains (e.g., *Streptococcus*), pairs (e.g., *Neisseria*), or tetrads (e.g., *Micrococcus*).
- **Bacilli** may appear as single rods, in chains, or in palisades (e.g., *Corynebacterium*).

## 5. Motility

- The ability of microorganisms to move is another important morphological trait. Some microorganisms have flagella or cilia for movement, while others are non-motile.
- **Flagella arrangement**:
  - **Monotrichous**: One flagellum at one end.
  - **Lophotrichous**: A tuft of flagella at one end.
  - **Amphitrichous**: A flagellum at both ends.
  - **Peritrichous**: Flagella distributed all over the surface.
- Motility can be observed using techniques such as the **hanging drop method** or by inoculating in motility agar media.

## 6. Colony Morphology

- When microorganisms grow on solid media (like agar plates), they form colonies. The characteristics of these colonies can aid in identification:
- **Size**: Colony size can range from tiny pinpoint colonies to large, spreading colonies.
- **Shape**: Colonies can be round, irregular, or filamentous.
- **Edge**: The edges of colonies may be smooth, wavy, or irregular.
- **Elevation**: Colonies may be flat, raised, or convex.
- **Color and Texture**: The color of the colony and the texture (e.g., smooth, rough, shiny, or matte) provide clues.
- **Opacity**: Colonies can be translucent, opaque, or transparent.

**7. Spore Formation**

- Some microorganisms, particularly certain bacteria and fungi, produce spores. The presence, type, and location of spores can help with identification:
- **Endospores**: These are highly resistant spores formed inside certain bacteria (e.g., *Bacillus* and *Clostridium* species).
- **Conidia and Sporangia**: Spore-bearing structures found in fungi, such as conidia in molds like *Aspergillus*.

**8. Other Features (e.g., Capsules, Pigments)**

- **Capsules**: Some bacteria have a gelatinous capsule surrounding their cell wall, which can be observed under a microscope using special stains (e.g., India ink stain).
- **Pigments**: Certain microorganisms produce pigments that give their colonies distinctive colors (e.g., *Serratia marcescens* produces a red pigment).

**9. Microscopic Structure**

- Examining the internal structure of microorganisms under a microscope can provide further information for identification. This includes the presence of organelles in eukaryotic cells (like fungi or protozoa) or internal structures like plasmids and inclusion bodies in bacteria.

**10. Special Staining Techniques**

- Certain microorganisms may require specialized staining techniques for better visualization of their morphology, such as:
- **Acid-fast staining**: Used for identifying mycobacteria (e.g., *Mycobacterium tuberculosis*), which retain the stain despite being washed with acid.
- **Capsule staining**: To visualize the capsule surrounding certain bacteria.

- **Identification of microorganisms using biochemical methods** involves testing the metabolic and chemical characteristics of microorganisms to determine their identity. These tests provide information about the enzymes a microorganism produces, the types of metabolic pathways it follows, and its ability to utilize or produce specific substances. Biochemical methods are often used in combination with morphological techniques to help identify microorganisms more accurately.

Here are some **common biochemical methods** for identifying microorganisms:

**1. Carbohydrate Fermentation Tests**

- Many bacteria ferment specific carbohydrates, producing either acid, gas, or both. These tests help determine which carbohydrates a microorganism can metabolize:
- **Glucose, Lactose, Sucrose Fermentation**: A microorganism is inoculated into a medium containing a specific sugar and phenol red as a pH indicator. If the organism ferments the sugar, the pH decreases (acid production), turning the medium yellow. Gas production (from fermentation) may also be observed in a Durham tube.
- **Triple Sugar Iron (TSI) Agar**: Used to test for the ability to ferment glucose, lactose, and sucrose, and for hydrogen sulfide production.

**2. Enzyme Activity Tests**

- Microorganisms produce specific enzymes that allow them to break down substrates. Testing for the presence or absence of these enzymes can help identify microorganisms:
- **Catalase Test**: Catalase is an enzyme that breaks down hydrogen peroxide into water and oxygen. The addition of hydrogen peroxide to a bacterial culture that produces catalase results in bubbling (oxygen release). This test differentiates between **catalase-positive** bacteria (e.g., *Staphylococcus*) and **catalase-negative** bacteria (e.g., *Streptococcus*).

- **Oxidase Test**: Detects the presence of cytochrome c oxidase, an enzyme involved in the electron transport chain. A positive result (color change) indicates an **oxidase-positive** organism, such as *Pseudomonas*.
- **Urease Test**: Detects the enzyme urease, which breaks down urea into ammonia and carbon dioxide. A pink color indicates urease activity, suggesting microorganisms like *Proteus* that produce urease.
- **Coagulase Test**: The enzyme coagulase causes plasma to clot. This test is particularly useful for differentiating *Staphylococcus aureus* (coagulase-positive) from other staphylococci (coagulase-negative).

**3. Amino Acid Decarboxylation**

- In this test, specific amino acids are added to an agar medium. If the microorganism decarboxylates the amino acid (removes a carboxyl group), it produces an alkaline by-product, which causes a color change in the medium (e.g., from yellow to purple). Common tests include **lysine decarboxylation** and **ornithine decarboxylation**.

**4. Indole Production Test**

- This test determines the ability of an organism to break down the amino acid tryptophan into indole. After adding Kovac’s reagent to a culture, a red color indicates **indole-positive** organisms, such as *Escherichia coli*.

## 5. Methyl Red and Voges-Proskauer (MR-VP) Tests

- These tests assess the type of fermentation pathway a microorganism follows:
- **Methyl Red (MR) Test**: Detects mixed acid fermentation. If the microorganism produces a significant amount of acid, the pH will drop, and the addition of methyl red will turn the solution red, indicating **MR-positive** organisms (e.g., *Escherichia coli*).
- **Voges-Proskauer (VP) Test**: Detects the production of acetoin (a precursor of butylene glycol). A positive result is indicated by a red color change after the addition of reagents, identifying organisms like *Enterobacter*.

## 6. Citrate Utilization Test

- Some microorganisms can utilize citrate as their sole carbon source. In this test, an organism is inoculated into a medium containing citrate. If the organism utilizes citrate, it will produce alkaline by-products that change the color of the medium to blue, indicating **citrate-positive** organisms (e.g., *Klebsiella* and *Enterobacter*).

## 7. Nitrate Reduction Test

- This test determines whether microorganisms can reduce nitrate ($NO_3^-$) to nitrite ($NO_2^-$) or further to nitrogen gas ($N_2$). A color change after adding reagents indicates the presence of nitrate reduction.

## 8. Hydrogen Sulfide ($H_2S$) Production Test

- Some bacteria produce hydrogen sulfide as a by-product of metabolism. In the **SIM medium** (Sulfide, Indole, Motility), if the bacteria produce $H_2S$, the medium will turn black due to the reaction of $H_2S$ with iron salts in the medium. This is commonly seen in **$H_2S$-positive** bacteria such as *Salmonella*.

## 9. API (Analytical Profile Index) Systems

- API systems are commercial kits used for the rapid identification of bacteria based on multiple biochemical reactions. Each test in the kit measures different metabolic activities, and a numerical profile is generated, which can be compared to a database for identification.

## 10. Metabolic Pathway Tests

- Some microorganisms exhibit specific metabolic pathways that can be used for identification:
- **Fermentation of specific sugars** (e.g., mannitol, arabinose) or the ability to produce specific metabolites (e.g., acetoin, ethanol).
- **Phenylalanine deaminase test**: This test checks for the ability to degrade phenylalanine to phenylpyruvic acid, which is detected by adding ferric chloride.

## 11. Saturation of Specific Growth Media

- The ability of microorganisms to grow in the presence of specific compounds or in selective media can also be used for identification. Examples include:
- **MacConkey agar**: Selective for Gram-negative bacteria and differentiates them based on lactose fermentation.
- **Sabouraud agar**: Used for the cultivation of fungi, especially yeasts and molds.
- **Enterotube System**: A commercially available, multi-test system that helps identify Gram-negative bacteria by analyzing several biochemical reactions at once.

- **Identification of microorganisms using molecular techniques** involves analyzing the genetic material (DNA or RNA) of microorganisms to identify and characterize them. These techniques are highly accurate, rapid, and can identify microorganisms at the species or even strain level, making them crucial for clinical diagnostics, research, and epidemiological studies. Molecular techniques often overcome the limitations of traditional culture-based methods, such as slow growth and difficulty in culturing certain microorganisms.
- Here are some **common molecular techniques** used for microorganism identification:

## 1. Polymerase Chain Reaction (PCR)

- **PCR** is one of the most widely used molecular techniques for microorganism identification. It amplifies specific DNA sequences, allowing the detection of microorganisms even if they are present in very low numbers.
- **Targeted PCR**: Involves amplifying specific genes or regions of the genome that are unique to a microorganism. Common targets include:
  - **16S rRNA gene**: Often used for bacterial identification, as it contains conserved and variable regions that can differentiate between species.
  - **ITS region**: The Internal Transcribed Spacer (ITS) region is commonly used for fungal identification.
- **Multiplex PCR**: Simultaneously amplifies multiple targets in a single reaction, allowing for the detection of several pathogens in one test.

## 2. Real-Time PCR (qPCR)

- **Quantitative PCR (qPCR)** is an advanced form of PCR that not only amplifies DNA but also quantifies it in real time. This technique can be used for:
- **Quantification of microbial load** in clinical samples (e.g., viral load measurement in HIV or COVID-19 infections).
- **Detection of specific microorganisms** by measuring the amplification of specific target sequences in real time, offering high sensitivity and specificity.

## 3. DNA Sequencing

- **DNA sequencing** involves determining the exact nucleotide sequence of a microorganism's DNA. This method provides the most precise identification and is used for species-level identification and characterization.
- **Sanger Sequencing**: Traditional method for sequencing smaller DNA fragments, such as 16S rRNA or ITS genes, for bacterial and fungal identification.
- **Next-Generation Sequencing (NGS)**: High-throughput sequencing technology that allows for the sequencing of entire genomes or metagenomes. It provides a comprehensive view of microbial diversity in environmental, clinical, or clinical microbiome samples. NGS is particularly useful in identifying unculturable microorganisms and conducting metagenomic studies.

**4. Ribotyping**

- **Ribotyping** is a molecular technique based on the analysis of the 16S or 23S rRNA gene sequence. It helps in the identification of bacterial species and subtyping of strains by comparing the ribosomal DNA (rDNA) patterns of different isolates. This method can be used for epidemiological surveillance and tracking the spread of pathogens.

**5. Restriction Fragment Length Polymorphism (RFLP)**

- **RFLP** involves digesting DNA with restriction enzymes, separating the resulting fragments by gel electrophoresis, and analyzing the patterns (fingerprints) of the fragments. This method is used for:
- **Genetic fingerprinting** of microorganisms.
- **Identifying bacterial or fungal strains** and differentiating closely related species.

**6. FISH (Fluorescence In Situ Hybridization)**

- **FISH** is a molecular technique used to detect specific microorganisms or groups of microorganisms within a sample using fluorescently labeled DNA or RNA probes. The probes are complementary to specific sequences in the target microorganism’s genome. FISH allows for:
- **In situ visualization** of microorganisms in clinical or environmental samples.
- **Identification of species or genera** based on ribosomal RNA (rRNA) sequences.
- **7. Metagenomic Sequencing**
- **Metagenomic sequencing** involves sequencing the collective genome of all microorganisms in a given sample (e.g., soil, water, human microbiome). This approach does not require culturing the organisms and can identify a wide variety of microorganisms, including rare and unculturable species.
- **Shotgun sequencing**: Sequences all DNA fragments in a sample, providing a comprehensive view of the microbial community.
- **Amplicon sequencing**: Targets specific genes (such as the 16S rRNA gene) to characterize the microbial composition of a sample.

## 8. DNA Microarrays

- **DNA microarrays** use a large number of probes fixed on a solid surface (such as a chip) to detect and identify microorganisms based on their genomic sequences. The microorganisms in the sample hybridize to complementary probes on the array, and the pattern of hybridization can be used to identify them.
- **Pathogen detection arrays**: Used to detect a broad range of pathogens in a sample by testing for the presence of specific genes.
- **Microbial community profiling**: Can be used for studying the diversity of microbial communities in environmental or clinical samples.

## 9. Whole Genome Sequencing (WGS)

- **Whole Genome Sequencing** involves sequencing the entire genome of a microorganism. It is used for the most accurate and detailed identification and characterization of pathogens, including:
- **Antimicrobial resistance profiling**: Identifying resistance genes in bacterial pathogens.
- **Comparative genomics**: Comparing genomes of different strains or species to study their evolution, virulence factors, and potential for infection.
- **Epidemiological studies**: Tracking outbreaks of infections by comparing genome sequences from various sources.

## 10. Amplified Fragment Length Polymorphism (AFLP)

- **AFLP** is a method that combines restriction enzyme digestion of DNA with selective amplification of DNA fragments. It is used to generate DNA fingerprints that can be used to identify microorganisms, study genetic diversity, and investigate clonal relationships among strains.

## 11. Single Nucleotide Polymorphism (SNP) Analysis

- **SNP analysis** involves detecting genetic variations at specific positions in the genome (single nucleotide polymorphisms). It is used for:
- **Subtyping microorganisms** at a high resolution.
- **Identifying pathogen strains** in epidemiological studies.
- **Assessing genetic variation** within microbial populations.

**Advantages of Molecular Techniques:**

- **High Sensitivity and Specificity**: Molecular methods can detect microorganisms at very low concentrations and with high accuracy.
- **Ability to Identify Uncultured Microorganisms**: Many microorganisms that are difficult or impossible to culture can still be identified through their DNA or RNA sequences.
- **Rapid Results**: Molecular techniques can provide results much faster than traditional culture-based methods, often within hours.
- **Identification at the Species or Strain Level**: These techniques can differentiate between closely related species or strains, which is essential for clinical diagnostics and epidemiology.
- **Comprehensive Profiling**: Techniques like metagenomics allow the identification of entire microbial communities, which is useful in environmental and human microbiome studies.