Tutorial session 3: Common staining techniques in microbiology

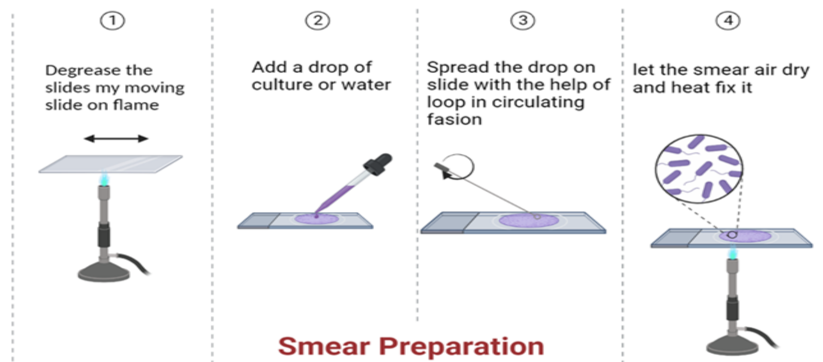
I- Introduction

Most bacteria lack intrinsic colour, which is why the application of a staining reagent is necessary to make them visible under a microscope. Once stained, bacteria can be studied in terms of their shape, size, and arrangement. The preparation of high-quality smears is a prerequisite for staining procedures.

II- Preparation of fixed smears

A bacterial smear is a **thin layer** of bacteria spread onto a glass slide and fixed by heat or chemical methods to ensure the cells adhere firmly to the slide during staining. This step is essential for maintaining the integrity of the bacterial structure and preventing the sample from washing off during the staining process. The smear should be prepared as soon as possible after sampling to avoid changes in bacterial morphology or viability.

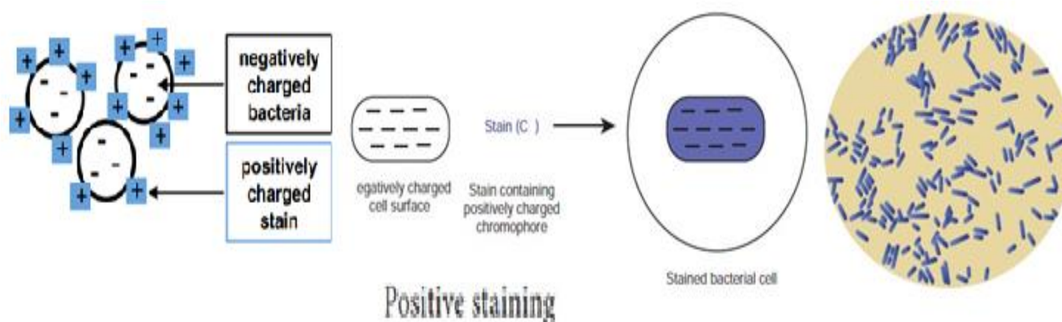
Depending on the source, the smear can be prepared from either a solid medium, such as an agar plate, or a liquid medium, such as a broth culture. Proper preparation of the smear is critical for achieving clear and accurate microscopic observations, as it ensures an even distribution of bacteria and minimizes artifacts that could interfere with interpretation.



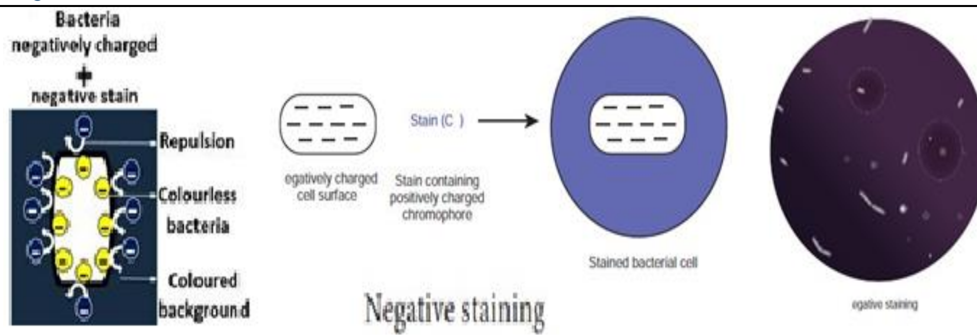
III. Staining techniques

The surface and cytoplasm of bacterial cells, as well as their DNA, RNA, proteins, and chromosome, are negatively charged. This property allows them to interact with dyes in different ways, depending on the staining technique used:

- **In direct/positive staining**, a positively charged dye is applied, which adheres to the negatively charged cell structures.



- **In negative staining**, an acidic dye (negatively charged) is used, repelling the cell surface and staining the background instead.



Staining techniques can be broadly categorized into **simple staining** and **differential staining**, each serving specific purposes in microbiological studies.

III. 1. Simple Staining

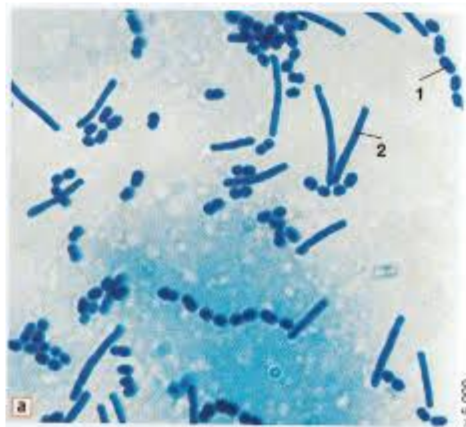
The coloration of bacteria by applying a single staining solution to a fixed smear is called **simple staining**. It is also referred to as **monochrome staining** because only one dye is used.

Simple staining can be:

III.1.1. Positive staining

It stains objects (in a single **colour**) **but not** the background. Basic dyes with positively charged groups, like methylene blue, crystal violet, safranine and malachite green are used to determine the shape, size and arrangement of bacteria.

All organisms in a sample will exhibit the same colour, even if the sample contains more than one type of organism.



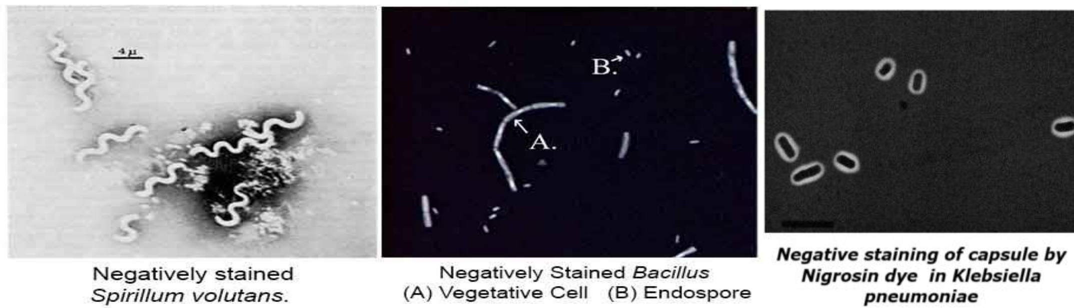
Simple staining using methylene blue

III.1.2. Indirect/negative staining

Negative staining colours the entire preparation **except** the elements to be observed. This technique uses acidic dyes, which carry negatively charged groups and stain only the **background**. It is particularly useful for visualizing the morphology, size, and arrangement of delicate bacterial cells, such as *Spirillum* or *Treponema*, without distorting their structure.

Unlike other staining methods, negative staining does not colour the bacterial cells themselves. Instead, the cells appear as colourless bodies against a dark background.

An acidic dye, such as India ink or nigrosine, is used. Negative staining is an acidic staining method, meaning the dye readily donates a hydrogen ion (proton), causing the chromophore of the dye to become negatively charged. Since the surface of most bacterial cells is also negatively charged, the cell surface repels the dye. As a result, bacteria appear as bright spots against a dark background.



Negatively stained *Spirillum volutans.*

Negatively Stained *Bacillus* (A) Vegetative Cell (B) Endospore

Negative staining of capsule by Nigrosin dye in Klebsiella pneumoniae

III.2. Differential Staining

Differential staining techniques are used to study bacterial morphology and to classify bacterial cells into distinct groups. Differential staining methods typically require multiple dyes and several staining steps. When used for bacterial identification, differential staining can be combined with other methods.

III.2.1. Gram staining

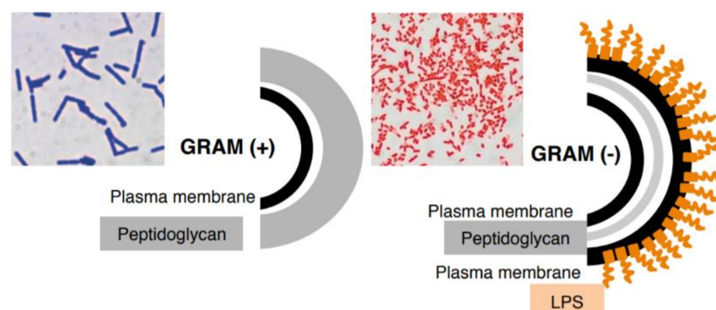
The Gram stain was developed by **Hans Christian Gram** (a Danish physician) in 1884. It is the most widely employed staining method in bacteriology. This staining procedure particularly defines two bacterial groups, Gram-positive (positive by gram method), and Gram-negative (negative by gram method). This is the starting point for the bacterial identification procedure.

It allows bacteria to be differentiated based on **shape** (cocci, bacilli, etc.), **arrangement** (pairs, tetrads, clusters, chains, etc.) and **affinity for dyes** (Gram-positive or Gram-negative).

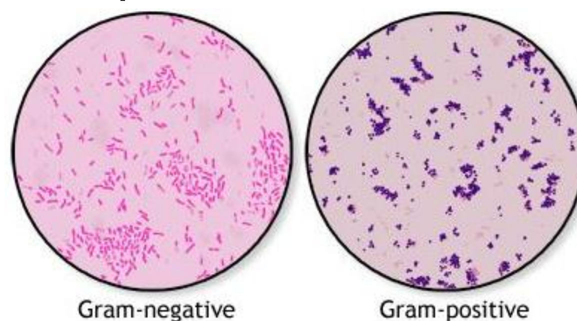
Principle

Gram-positive bacteria have thick, dense, relatively, non-porous walls, while gram-negative have thin walls with lipid-rich membranes (due to the outer membrane) and a fine layer of peptidoglycan. The alcohol in the decolorizing agent extracts the lipids, making the cell wall of Gram-negative bacteria more porous and unable to retain the crystal violet-iodine complex, thus decolorizing the bacteria.

The thicker peptidoglycan layer with a higher degree of cross-linking traps the crystal violet-iodine complex more effectively, making the Gram-positive cell wall less susceptible to decolorization.



- Gram Positive bacteria: **blue** or **purple color**
- Gram Negative bacteria: **red or pink color**



III.2.2. Ziehl-Neelsen (ZN) Staining

The Ziehl-Neelsen staining technique is one of the most widely used methods for detecting *Mycobacterium tuberculosis*, also known as Koch's bacillus, the bacterium responsible for tuberculosis. It is the method of choice for sputum smear microscopy because it consistently provides reliable results without requiring specialized equipment.

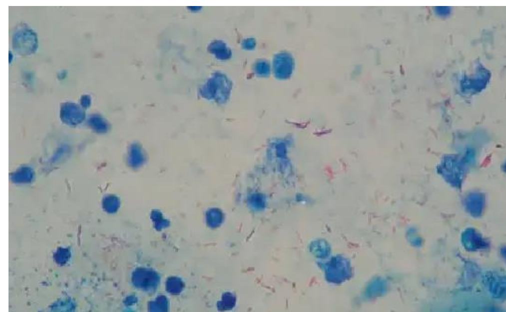
Principle

The Ziehl-Neelsen staining technique relies on the presence of mycolic acids in the cell walls of **acid-fast bacteria**, which is the cytological basis for this method. Mycolic acid gives these bacteria a greater affinity for the primary stain and resistance to decolorization by an acid-alcohol solution.

Carbol fuchsin is used as the primary stain because it is lipid-soluble and penetrates the waxy cell wall of acid-fast bacteria. The staining process is enhanced by heating the preparation until steam is produced, which melts the wax and allows the dye to penetrate the cell effectively.

After staining, an acid-alcohol solution is used to decolorize non-acid-fast cells, which lose the primary stain. A counterstain, such as methylene blue, is then applied to provide contrast. Once the staining process is complete, acid-fast bacteria appear red-violet against a blue background, while non-acid-fast cells and background material are stained blue.

This principle makes the Ziehl-Neelsen staining method highly effective for identifying acid-fast bacteria, such as *Mycobacterium tuberculosis*, in clinical samples.

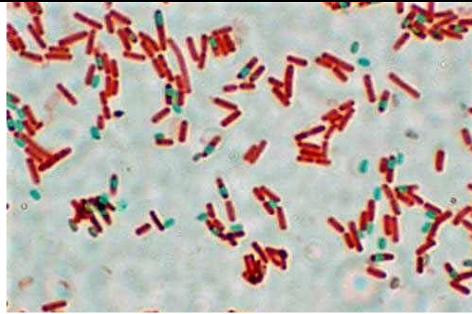


III.2.3. Spore staining with Malachite Green (Schaeffer-Fulton method)

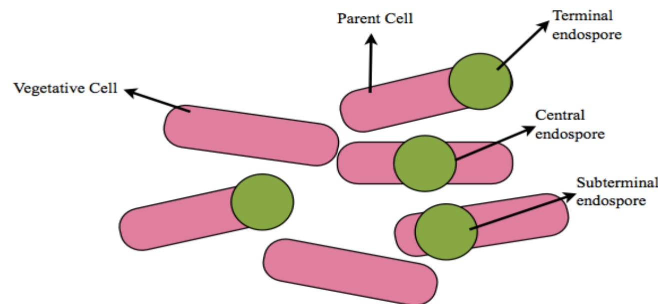
This method is highly effective for visualizing endospores, which are resistant structures formed by certain bacteria under **stressful conditions**. It is particularly useful for identifying species such as *Clostridium* and *Bacillus*, which are clinically and environmentally significant.

Principle

In the Schaeffer-Fulton method using malachite green, the primary dye (malachite green) is forced into the spore by heating the bacterial emulsion. Malachite green is water-soluble and has a low affinity for cellular material, allowing vegetative cells to be decolorized with water. Safranin is then applied as a counterstain to color the decolorized vegetative cells. At the end of the staining process, vegetative cells appear pink, while endospores appear dark green.



Spores may be located in the middle of the cell, at the end of the cell, or between the end and middle of the cell. Spore shape may also be of diagnostic use. Spores may be spherical or elliptical.



III.2.4. Flagella Staining (Rhodes method)

Observing flagella under an optical microscope requires special staining techniques to thicken and highlight these delicate structures. The Rhodes method is commonly used for this purpose. This method is essential for studying bacterial motility and flagellar arrangement.

Principle

Flagella are too thin and delicate to be seen under a light microscope without treatment. The Rhodes method uses a special stain called **flagella stain**, which contains a mordant. This mordant **enhances** staining, and a colloidal substance **is used** to thicken the flagella, making them visible. This helps to observe the various arrangements of flagella.

Bacterial cells appear pink which are surrounded by deeply stained flagella; the flagella may be monotrichous, amphitrichous, peritrichous, or lophotrichous. Flagella are much longer than the cell and often look like faint hairs.

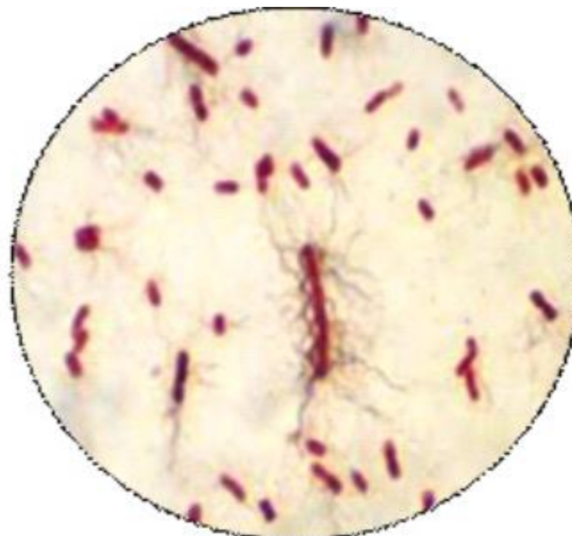


Fig. Flagella Staining