Microbiology Laboratory

Lab 5 : Staining

Gram Staining

Gram staining is a differential bacterial staining technique used to differentiate bacteria into Gram Positive and Gram-Negative types according to their cell wall composition. It is the most widely used and the most important staining technique in bacteriology, especially in medical bacteriology. It is generally the first test performed on bacteria during their identification and observation process. This staining technique uses two stains; crystal violet as primary stain and safranine as a counterstain. Those bacteria with Gram-positive cell walls will retain primary stain and appear violet or purple. These bacteria are termed Gram-Positive bacteria. The other group of bacteria with Gram-Negative cell wall will lose primary stain and take up the counterstain and appears pink or red under the microscope. These bacteria are called Gram-Negative bacteria. Using this staining technique, bacteria can be differentiated into two groups hence; it is called the differential staining technique.

History of Gram Staining:

This technique was introduced in 1884 by the Danish Bacteriologist **Hans Christian Gram** . He developed this staining technique to identify bacteria causing pneumonia. Later it became a popular method to classify bacteria into Gram Positive and Gram-Negative types.

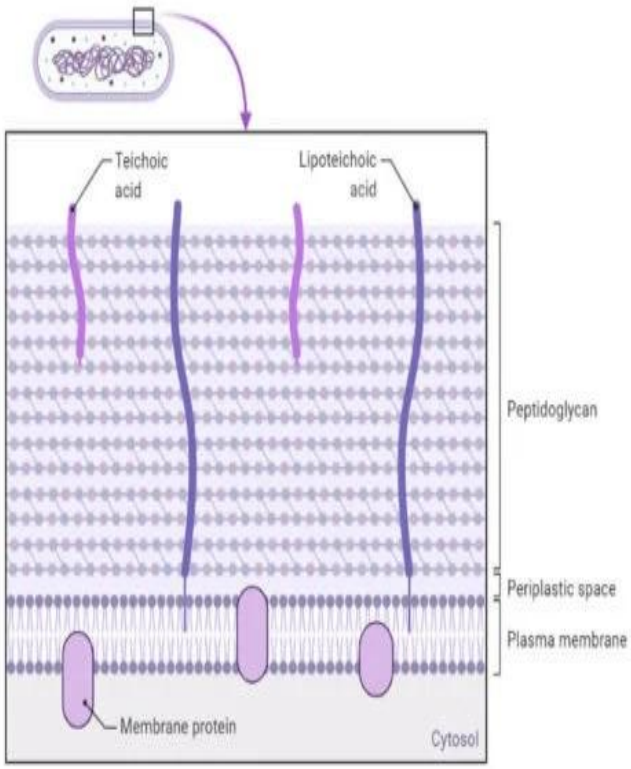
Gram Staining Objectives:

- To differentiate bacteria into Gram-Positive and Gram-Negative.
- To study the morphological structure of bacteria.

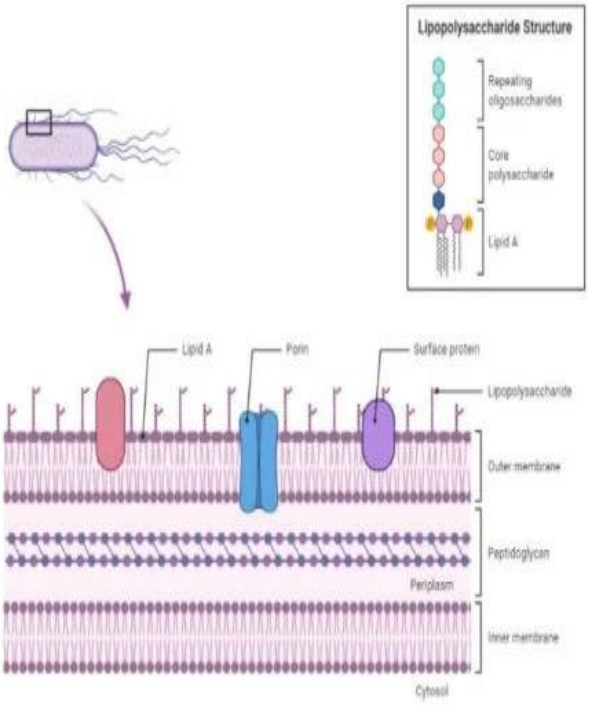
Gram Staining Principle:

Gram staining and differentiation are based on the differences in cell wall structure and composition of bacteria. Bacteria having cell walls with a thick layer of peptidoglycan will resist decolorization of primary stain and appear violet or purple. Bacteria having a thin peptidoglycan layer with lesser cross-linkage lose primary stain during decolorizing and gain counter stain appearing pink or red. When counterstain, positively charged safranin, is added, it interacts with the free negatively charged components in Gram-Negative cell wall and membrane and bacteria becomes pink/red. Whereas, there is no space to enter inside the dehydrated Gram-Positive cell wall due to CVI complex

and dehydration. Hence, safranin can't stain them red or pink and Gram-Positive bacteria reveal the purple or violet color.



Gram-Positive Bacteria Cell Wall Structure



Gram-Negative Bacteria Cell Wall Structure

Gram Staining Requirements:

- Sample bacterial colonies or suspension
- Gram Staining Kit (Reagents)
- Glass slide
- Inoculating loop
- Bunsen burner
- Staining rack
- Wash bottle (or Tap water)
- Microscope with 100X objective lens (compound microscope).

Gram Stain Reagents:

1. Primary Stain (Crystal Violet)
2. Mordant (Gram's Iodine)
3. Decolorizing Solution
4. Counter Stain (Safranin)

Gram Staining Protocol:

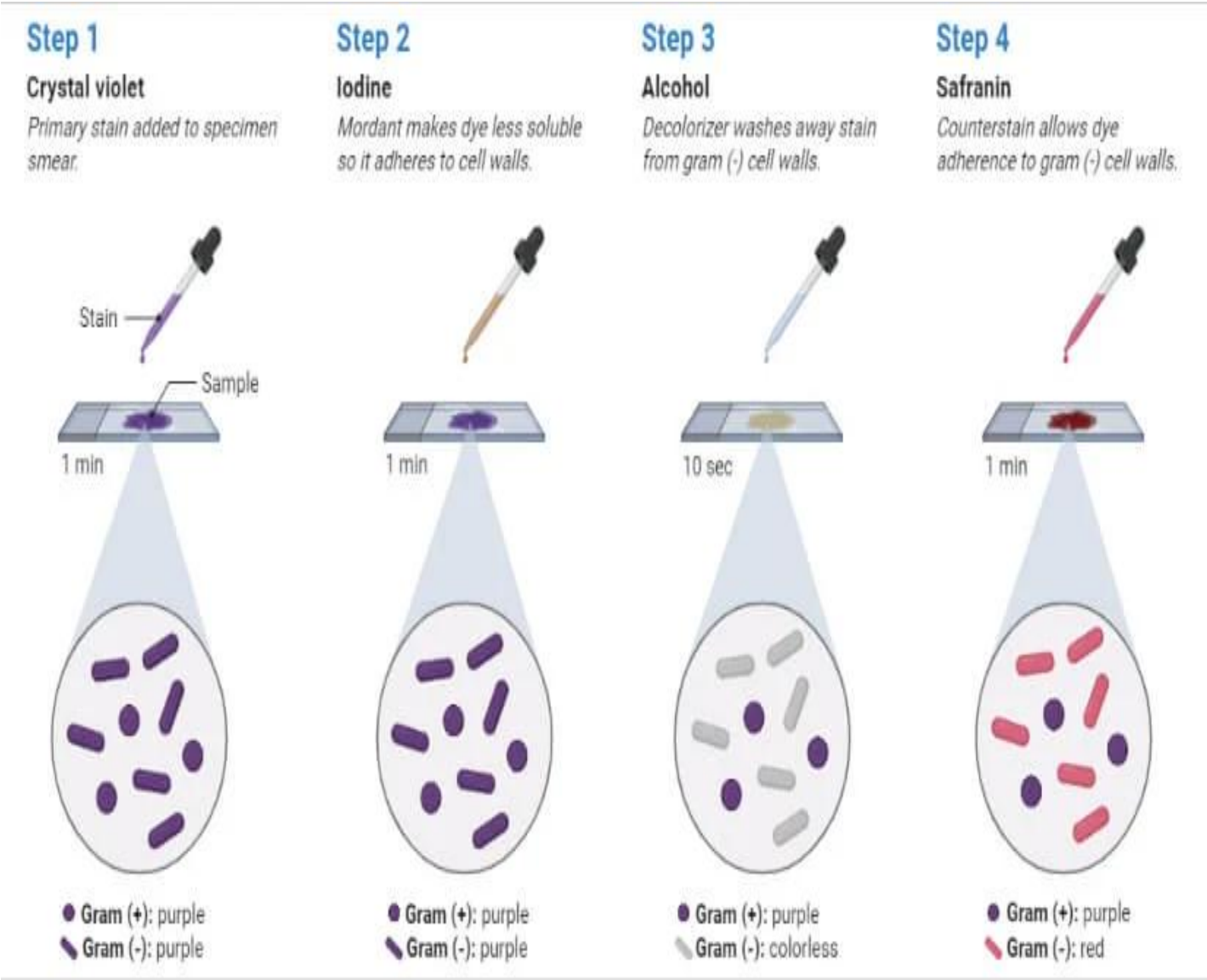
1. Flood crystal violet solution over fixed smear
2. After 30 – 60 seconds, pour off the CV solution and rinse with gentle running water.
3. Flood the Gram's Iodine solution over the smear
4. Leave the iodine solution for 30 – 60 seconds and pour off the excess iodine and rinse with gentle running water
5. Shake off the excess water over the smear
6. Decolorize the smear by passing the decolorizing solution till the solution runs down in clear form. Alternatively, add a few drops of decolorizing solution and shake gently and rinse with distilled water after 5 seconds.
7. Rinse with distilled water to wash decolorizer
8. Shake off the excess water over the smear
9. Pour counter stain over the smear
10. Leave for 30 – 60 seconds and wash with gentle running water
11. Air dry or blow-dry the smear.

Examples of Gram-positive bacteria:

1. **Gram-positive cocci**– *Staphylococcus spp., Streptococcus spp., Enterococcus spp*., etc.
2. **Gram-positive bacilli**– *Bacillus spp., Clostridium spp., Lactobacillus spp., Streptomyces spp.* and other Actinobacteria, *Listeria spp., Corynebacterium spp*., etc.

Examples of Gram-Negative bacteria:

1. **Gram negative cocci**– *Neisseria spp., Moraxella spp., Acinetobacter spp*. etc
2. **Gram negative bacilli-** *E. coli, Klebsiella spp., Salmonella spp., Shigella spp., Pseudomonas spp., Proteus spp.,* etc.



Gram-Positive bacteria appear violet or purple.

Gram-Negative bacteria appear pink or red.

Differential stain & Special stain

In their natural state, most of the cells and microorganisms that we observe under the microscope lack color and contrast. This makes it difficult, if not impossible, to detect important cellular structures and their distinguishing characteristics without artificially treating specimens. We have already alluded to certain techniques involving stains and fluorescent dyes, and in this section we will discuss specific techniques for sample preparation in greater detail. Indeed, numerous methods have been developed to identify specific microbes, cellular structures, DNA sequences, or indicators of infection in tissue samples, under the microscope. Here, we will focus on the most clinically relevant techniques.

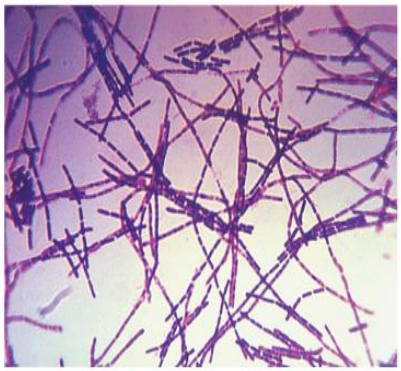
Preparing Specimens for Light Microscopy

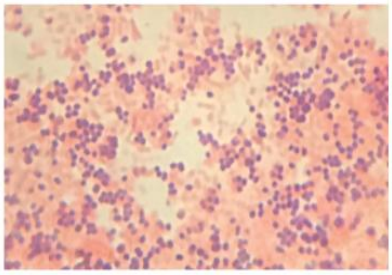
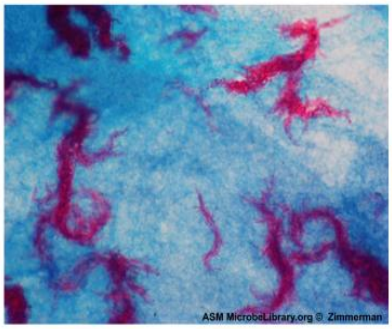
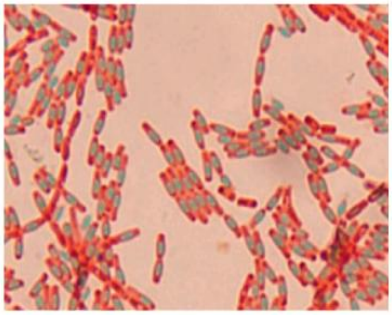
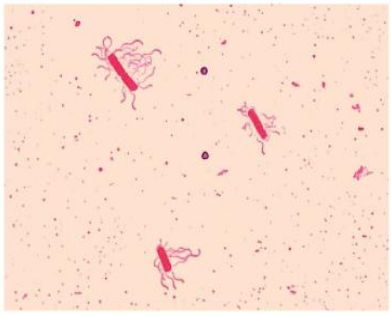
In clinical settings, light microscopes are the most commonly used microscopes. There are two basic types of preparation used to view specimens with a light microscope: wet mounts and fixed specimens.

The simplest type of preparation is the wet mount, in which the specimen is placed on the slide in a drop of liquid. Some specimens, such as a drop of urine, are already in a liquid form and can be deposited on the slide using a dropper. Solid specimens, such as a skin scraping, can be placed on the slide before adding a drop of liquid to prepare the wet mount. Sometimes the liquid used is simply water, but often stains are added to enhance contrast. Once the liquid has been added to the slide, a coverslip is placed on top and the specimen is ready for examination under the microscope. The second method of preparing specimens for light microscopy is fixation. The "fixing" of a sample refers to the process of attaching cells to a slide. Fixation is often achieved either by heating (heat fixing) or chemically treating the specimen. In addition to attaching the specimen to the slide, fixation also kills microorganisms in the specimen, stopping their movement and metabolism while preserving the integrity of their cellular components for observation. To heat-fix a sample, a thin layer of the specimen is spread on the slide (called a smear), and the slide is then briefly heated over a heat source. Chemical fixatives are often preferable to heat for tissue specimens. Chemical agents such as acetic acid, ethanol, methanol, formaldehyde (formalin), and glutaraldehyde can denature proteins, stop biochemical reactions, and stabilize cell structures in tissue samples.

In addition to fixation, staining is almost always applied to color certain features of a specimen before examining it under a light microscope. Stains, or dyes, contain salts made up of a positive ion and a negative ion. Depending on the type of dye, the positive or the negative ion may be the chromophore (the colored ion); the other, uncolored ion is called the counterion. If the chromophore is the positively charged ion, the stain is classified as a basic dye; if the negative ion is the chromophore, the stain is considered an acidic dye. Dyes are selected for staining based on the chemical properties of the dye and the specimen being observed, which determine how the dye will interact with the specimen. In most cases, it is preferable to use a positive stain, a dye that will be absorbed by the cells or organisms being observed, adding color to objects of interest to make them stand out against the background. However, there are scenarios in which it is advantageous to use a negative stain, which is absorbed by the background but not by the cells or organisms in the specimen. Negative staining produces an outline or silhouette of the organisms against a colorful background.

Some staining techniques involve the application of only one dye to the sample; others require more than one dye. In simple staining, a single dye is used to emphasize particular structures in the specimen. A simple stain will generally make all of the organisms in a sample appear to be the same color, even if the sample contains more than one type of organism. In contrast, differential staining distinguishes organisms based on their interactions with multiple stains. In other words, two organisms in a differentially stained sample may appear to be different colors. Differential staining techniques commonly used in clinical settings include Gram staining, acid-fast staining, endospore staining, flagella staining, and capsule staining.

SIMPLE STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Basic stains	Methylene blue, crystal violet, malachite green, basic fuchsin, carbolfuchsin, safranin	Stain negatively charged molecules and structures, such as nucleic acids and proteins	Positive stain	
Acidic stains	Eosin, acid fuchsin, rose bengal, Congo red	Stain positively charged molecules and structures, such as proteins	Can be either a positive or negative stain, depending on the cell's chemistry.	
Negative stains	India ink, nigrosin	Stains background, not specimen	Dark background with light specimen	

DIFFERENTIAL STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Gram stain	Uses crystal violet, Gram's iodine, ethanol (decolorizer), and safranin	Used to distinguish cells by cell-wall type (gram-positive, gram-negative)	Gram-positive cells stain purple/violet. Gram-negative cells stain pink.	
Acid-fast stain	After staining with basic fuchsin, acid-fast bacteria resist decolorization by acid-alcohol. Non acid-fast bacteria are counterstained with methylene blue.	Used to distinguish acid-fast bacteria such as *M. tuberculosis*, from non–acid-fast cells	Acid-fast bacteria are red; non–acid-fast cells are blue.	
Endospore stain	Uses heat to stain endospores with malachite green (Schaeffer-Fulton procedure), then cell is washed and counterstained with safranin.	Used to distinguish organisms with endospores from those without; used to study the endospore.	Endospores appear bluish-green; other structures appear pink to red.	
Flagella stain	Flagella are coated with a tannic acid or potassium alum mordant, then stained using either pararosaline or basic fuchsin.	Used to view and study flagella in bacteria that have them.	Flagella are visible if present.	
Capsule stain	Negative staining with India ink or nigrosin is used to stain the background, leaving a clear area of the cell and the capsule. Counterstaining can be used to stain the cell while leaving the capsule clear.	Used to distinguish cells with capsules from those without.	Capsules appear clear or as halos if present.	