4.1: Introduction to Staining

Learning Objectives

- Describe the differences between simple staining and differential staining techniques.
- Discuss how to prepare a bacterial smear from cultured organisms.
- Distinguish between Gram-positive and Gram-negative bacteria.
- Describe the process of the Gram stain procedure.
- Use microscopy to examine Gram stained cells.
- Describe select special staining procedures and view examples of these under oil immersion.

Why do we have to stain bacteria?

Most types of cells do not have much natural pigment and are therefore difficult to see under the light microscope unless they are stained. Several types of stains are used to make bacterial cells more visible. In addition, specific staining techniques can be used to determine the cells’ biochemical or structural properties, such as cell wall type and presence or absence of endospores. This type of information can help scientists identify and classify microorganisms, and can be used by health care providers to diagnose the cause of a bacterial infection.
The Simple Stain

One type of staining procedure that can be used is the **simple stain**, in which only one stain is used, and all types of bacteria appear as the color of that stain when viewed under the microscope. Some stains commonly used for simple staining include crystal violet, safranin, and methylene blue. Simple stains can be used to determine a bacterial species’ morphology and arrangement, but they do not give any additional information. Living bacteria are almost colorless, and do not present sufficient contrast with the water in which they are suspended to be clearly visible. The purpose of staining is to increase the contrast between the organisms and the background so that they are more readily seen in the light microscope. In a simple stain, a bacterial smear is stained with a solution of a single dye that stains all cells the same color without differentiation of cell types or structures. The single dye used here in our lab is methylene blue, a basic stain. Basic stains, having a positive charge, bind strongly to negatively charged cell components such as bacterial nucleic acids and cell walls.

![Microscopic view of Bacillus (rod) shaped bacteria simple stained with crystal violet.](https://commons.wikimedia.org/wiki/File:...micrograph.jpg)

**Image 1**: Microscopic view of Bacillus (rod) shaped bacteria simple stained with crystal violet. Isolated and imaged by Muntasir Alam, University of Dhaka, Department of Microbiology in 2007. [https://commons.wikimedia.org/wiki/File:...micrograph.jpg](https://commons.wikimedia.org/wiki/File:...micrograph.jpg)
Watch Video 1: how to apply a simple stain

[Video URL]

The Gram Stain

Scientists will often choose to perform a **differential stain**, as this allows them to gather additional information about the bacteria they are working with. Differential stains use more than one stain, and cells will have a different appearance based on their chemical or structural properties. Some examples of differential stains are the Gram stain, acid-fast stain, and endospore stain. You will learn how to prepare bacterial cells for staining, and learn about the gram staining technique.

This very commonly used staining procedure was first developed by the Danish bacteriologist Hans Christian Gram in 1882 (published in 1884) while working with tissue samples from the lungs of patients who had died from pneumonia. Since then, the Gram stain procedure has been widely used by microbiologists everywhere to obtain important information about the bacterial species they are working with. Knowing the Gram reaction of a clinical isolate can help the health care professional make a diagnosis and choose the appropriate antibiotic for treatment.
Gram stain results reflect differences in cell wall composition. **Gram positive** cells have thick layers of a peptidoglycan (a carbohydrate) in their cell walls; **Gram negative** bacteria have very little. Gram positive bacteria also have teichoic acids, whereas Gram negatives do not. Gram negative cells have an outer membrane that resembles the phospholipid bilayer of the cell membrane. The outer membrane contains lipopolysaccharides (LPS), which are released as endotoxins when Gram negative cells die. This can be of concern to a person with an infection caused by a gram negative organism.

Image 2: Microscopic image of a Gram stain of mixed Gram-positive cocci (*Staphylococcus aureus* ATCC 25923, purple) and Gram-negative bacilli (*Escherichia coli* ATCC 11775, red). Magnification: 1,000. Image by Y Tambe. [https://commons.wikimedia.org/wiki/F...m_stain_01.jpg](https://commons.wikimedia.org/wiki/F...m_stain_01.jpg)

Figure 1 below shows the major differences between the Gram positive and Gram negative cell walls. The differences in the cell wall composition are reflected in the way the cells react with the stains used in the Gram stain procedure.

Gram stains are best performed on fresh cultures—older cells may have damaged cell walls and not give the proper Gram reaction. Certain species are known as **Gram-variable**, and so both Gram positive and Gram negative reactions may be visible on your slide.

**Poor staining technique** could lead to inaccurate results. One of the most important steps in Gram staining is the decolorizing step (use of alcohol/acetone). If the decolorizer is not left on long enough, then it will not be able to differentiate between Gram positive and Gram negative bacteria. This step uses decolorizer, made of an alcohol/acetone mixture. Its function in Gram negative bacteria is to remove the outer cell membrane and thin layer of peptidoglycan. The cell membrane is mostly made of lipids and are sensitive to alcohols. By dissolving these layers, the crystal violet-iodine complex is also removed, and thus Gram negatives are now able to take up the secondary stain, safranin, which is used in the last step of the Gram stain, staining them pinkish-red and differentiating between them and the Gram positives, who with their thick peptidoglycan layer has retained the primary stain, crystal violet, and appears purple/blue. On the flip side, if you use too much decolorizer, it can decolorize your sample on the slide, leading to loss of crystal violet (the primary stain)-iodine complex. The decolorizing step is sensitive because of the cell wall structure. Even Gram positive bacteria with their thick cell walls could become excessively decolorized, resulting in the loss of the
peptidoglycan layer and the crystal violet-iodine complex. When the use of the secondary stain, safranin, is applied in the last step, the Gram positive bacteria will pick up this stain and look reddish-pink instead of purple/blue. Watch video 2 for an example of this.

Another common mistake is in the preparation of the bacterial smear, which is in the first step of any staining procedure. This involves applying a thin film of bacteria on your microscope slide and then **heat fixing** it with either your bunsen burner, bacticinerator, or slide warmer. The main purpose of this step is to adhere the bacterial cells to the microscope slide (it also denatures the proteins and kills them too). If you forget to do this step, then the cells will be 'washed' off in all the subsequent steps of your staining process. You will literally have no cells on your slide to stain!

Although the vast majority of bacteria are either Gram positive or Gram negative, it is important to remember that not all bacteria can be stained with this procedure (for example, Mycoplasmas, which have no cell wall, stain poorly with the Gram stain).

![Gram positive and Gram negative cell walls](https://bio.libretexts.org/Courses/North_Carolina_State_University/MB352_General_Microbiology_Laboratory_2021_(Lee)/04...)}
Watch Video 2: Gram Stain Animation and discussion on what is happening at each step.

Watch Video 2: Gram stain animation with description of each step and interpretation of what is happening in each step by

Dr. G Bhanu Prakash Animated Medical Videos. (3:37) URL https://youtu.be/AZS2wb7pMo4
Watch Video 3: Gram staining procedure

Special Stains

There are a variety of staining procedures used to identify specific external or internal structures that are not found in all bacterial species, such as a capsule stain and a flagella stain. For images and more examples of specialized stains, see below and in the next section, 4.2 specialized bacterial staining techniques.

Capsule Stain

Some bacteria secrete a polysaccharide-rich structure external to the cell wall called a glycocalyx. If the glycocalyx is thin and loosely attached, it is called a slime layer; if it is thick and tightly bound to the cell, it is called a capsule. The glycocalyx can protect the cell from desiccation and can allow the cell to stick to surfaces like tissues in the body. They may also provide cells with protection against detection and phagocytosis by immune cells and contribute to the formation of a biofilm: in this way a glycocalyx can act as a virulence factor; (contributes to the ability of an organism to cause disease).
Capsules can be detected using a **negative staining** procedure in which the background (the slide) and the bacteria are stained, but the capsule is not stained. The capsule appears as a clear unstained zone around the bacterial cell. Since capsules are destroyed by heat, the capsule staining procedure is done without heat-fixing the bacteria.

### Silver Stain

**Flagella** (long whip-like structures used for bacterial motility) and some bacteria (e.g. **spirochetes**) are too thin to be observed with regular staining procedures. In these cases, a **silver stain** is used. Silver nitrate is applied to the bacteria along with a special mordant; the silver nitrate precipitates around the flagella or the thin bacteria, thus thickening them so they can be observed under the light microscope.