Bacterial staining

Visualization of microorganisms in the living state is quite difficult, not only because they are minute, but also because they are transparent and practically colorless when suspended in an aqueous medium. To study their properties and to divide microorganisms into specific groups for diagnostic purposes, biological stains and staining procedures in conjunction with light microscopy have become major tools in microbiology. The ability of a stain to bind to macromolecular cellular components such as proteins or nucleic acids depends on the electrical charge found on the chromogen portion, as well as on the cellular component to be stained. There are two types of stains (**dyes**):

Basic dyes -methylene blue, basic fuchsin, crystal violet, safranin, malachite green- have positively charged groups (usually some form of pentavalent nitrogen) and are generally sold as chloride salts. Basic dyes bind to negatively charged molecules like nucleic acids and many proteins. Because the surfaces of bacterial cells also are negatively charged, basic dyes are most often used in bacteriology.

Acid dyes -eosin, rose bengal, and acid fuchsin- possess negatively charged groups such as carboxyls (-COOH) and phenolic hydroxyls (-OH). Acid dyes, because of their negative charge, bind to positively charged cell structures.

To apply acidic or basic dyes, microbiologists use three kinds of staining techniques: **simple**, **differential**, and **special**.

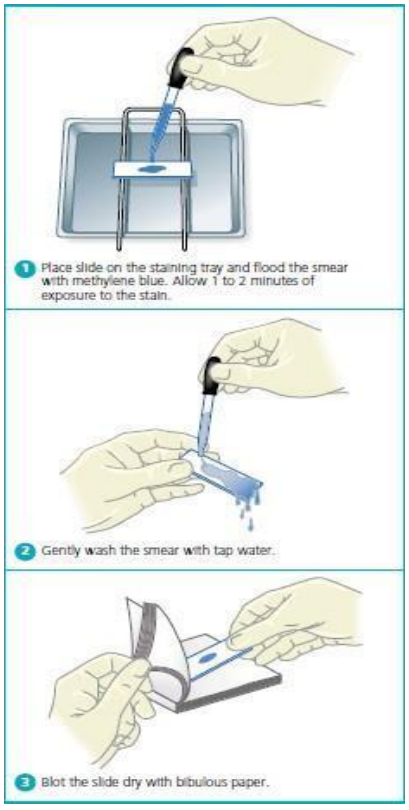
Preparing Smears for Staining

Most initial observations of microorganisms are made with stained preparations. **Staining** simply means coloring the microorganisms with a dye that emphasizes certain structures. Before the microorganisms can be stained, however, they must be **fixed** (attached) to the microscope slide. Fixing simultaneously kills the microorganisms and fixes them to the slide. It also preserves various parts of microbes in their natural state with only minimal distortion. When a specimen is to be fixed, a thin film of material containing the microorganisms is spread over the surface of the slide. This film, called a **smear,** is allowed to air dry. In most staining procedures the slide is then fixed by **Heat fixation**, where slides pass through the flame of a Bunsen burner several times, smear side up, or by **Chemical fixation** (covering the slide with

methanol for 1 minute). Stain is applied and then washed off with water; then the slide is blotted with absorbent paper. Without fixing, the stain might wash the microbes off the slide.

Simple staining

Principle: In simple staining, the bacterial smear is stained with a single basic dye, which produces a distinctive contrast between the organism and its background. Basic stains with a positive charge are preferred because bacterial nucleic acids and certain cell wall components carry a negative charge that strongly attracts and binds to the cationic chromogen. The purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. The most commonly used basic stains are methylene blue, crystal violet, and carbol fuchsin.



Procedure:

1- Prepare separate bacterial smears of the organisms by heat fixation.
2- Place slide on the staining tray and flood the smear with methylene blue. Allow 1 to 2 minutes of exposure to the stain.
3- Gently wash the smear with tap water.
4- Blot the slide dry with bibulous paper.

Differential stains

Unlike simple stains, differential stains use two contrasting stains and react differently with different kinds of bacteria, and thus can be used to distinguish them. The differential stains most frequently used for bacteria are the **Gram stain** and the **Acid-fast stain.**

Gram stain

The Gram stain was developed in 1884 by the Danish bacteriologist Hans Christian Gram. It is one of the most useful staining procedures because it classifies bacteria into two large groups: gram-positive and gram-negative.

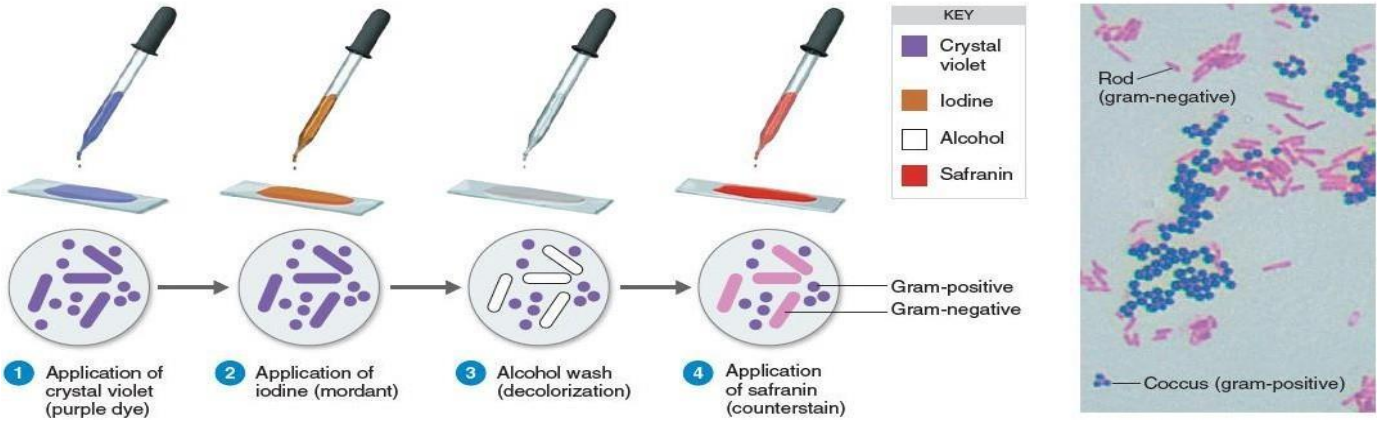
Procedure:

1- A heat-fixed smear is covered with a basic purple dye, usually crystal violet. Because the purple stain imparts its color to all cells, it is referred to as a **primary stain.**

2- After a short time (one minute), the purple dye is washed off, and the smear is covered with **iodine** (for one minute). When the iodine is washed off, both gram-positive and gram-negative bacteria appear dark violet or purple.
Note: **Iodine** reagent serves not only as a killing agent but also as a **mordant**, a substance that increases the cells' affinity for a stain.

3- Next, the side is washed with alcohol or an alcohol-acetone solution for 10–30 seconds. This solution is a **decolorizing agent,** which removes the purple from the cells of some species but not from others.

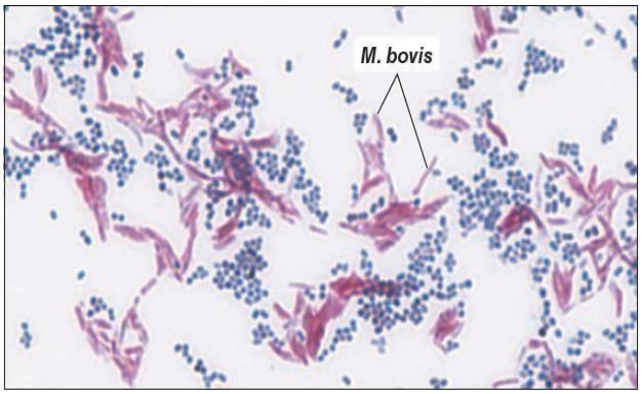
4- The alcohol is rinsed off, and the slide is then stained with **safranin** (for 45 seconds), a basic red dye. The smear is washed again by tap water, blotted dry, and examined microscopically.



Interpretation of results: The purple dye and the iodine combine in the cell wall and cytoplasm of each bacterium and color it dark violet or **purple**. Bacteria that retain this color after the alcohol has attempted to decolorize them are classified as **gram-positive;** bacteria that lose the dark violet or purple color after decolorization are classified as **gram-negative.** Because gram-negative bacteria are colorless after the alcohol wash, they are no longer visible. This is why the basic dye safranin is applied; it turns the gram-

negative bacteria **pink**. Stains such as safranin that have a contrasting color to the primary stain are called **counterstains**. Because gram-positive bacteria retain the original purple stain, they are not affected by the safranin counterstain.

Acid-fast stain (Ziehl-Neelsen method):



The characteristic difference between mycobacteria and other microorganisms is the presence of a thick, waxy (lipoidal) wall that makes penetration by stains extremely difficult. Therefore, another important differential stain (one that differentiates bacteria into distinctive groups) is the **acid-fast stain,** which binds strongly only to bacteria that have a waxy material in their cell walls. Microbiologists use this stain to identify all bacteria in the genus *Mycobacterium*, including the two important pathogens *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and *Mycobacterium leprae*, the causative agent of leprosy. This stain is also used to identify the pathogenic strains of the genus *Nocardia.* Bacteria in the genera *Mycobacterium* and *Nocardia* are acid-fast.

Procedure:

1- The red dye carbolfuchsin is applied to a fixed smear, and the slide is gently heated for 5 minutes. (Heating enhances penetration and retention of the dye.)
   Note: *Do not allow stain to evaporate, replenish stain as needed.*
2- Then the slide is cooled and washed with water.
3- The smear is next treated with **acid-alcohol** (3% HCl + 95% Ethanol), a decolorizer, adding the reagent drop by drop until the alcohol runs almost clear with a slight red tinge. That's will remove the red stain from bacteria that are not acid-fast.
   Note: The acid-fast microorganisms retain the pink or red color because the carbolfuchsin is more soluble in the cell wall lipids than in the acid alcohol. In non–acidfast bacteria, whose cell walls lack the lipid components, the carbolfuchsin is rapidly removed during decolorization, leaving the cells colorless.

4- The smear is then stained with a methylene blue counterstain (for 2 minutes). Non−acid-fast cells appear **blue** after the counterstain is applied.

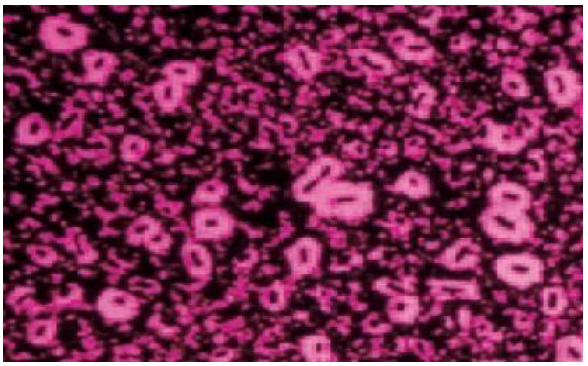
Special Stains:

Special stains are used to color parts of microorganisms, such as **capsules, endospores** and F**lagella**.

1- Negative Staining for Capsules

One of the simplest is **negative staining**. Many microorganisms contain a gelatinous covering called a **capsule.** In medical microbiology, demonstrating the presence of a capsule is a means of determining the organism′s virulence, the degree to which a pathogen can cause disease**.**

Procedure:



Bacteria are mixed with India ink or Nigrosin dye and spread out in a thin film on a slide. After air-drying (*Do not heat fix*), bacteria appear as lighter bodies in the midst of a blue-black background because ink and dye particles cannot penetrate either the bacterial cell or its capsule. The extent of the light region (halos) is determined by the size of the capsule and of the cell itself.

2- Endospore (Spore) Staining

Principle: An **endospore** is a special resistant, dormant structure formed within a cell that protects a bacterium from adverse environmental conditions. Endospores cannot be stained by ordinary methods, such as simple staining and Gram staining, because the dyes don′t penetrate the endospore′s wall**.** Microbiologists use this stain to identify dangerous spore-forming bacteria such as *Bacillus anthracis*, which causes anthrax, and certain *Clostridia* bacteria, which are the causative agents for tetanus, gas gangrene, food poisoning.

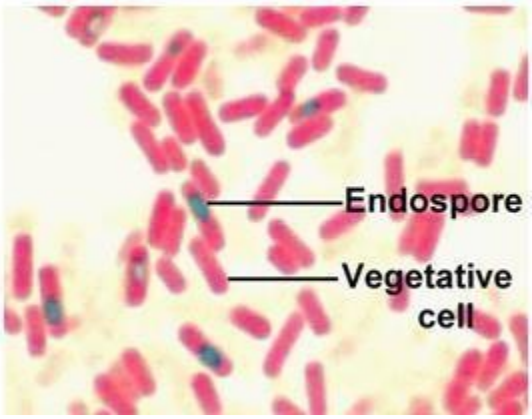
Procedure: The most commonly used endospore staining procedure is the ***Schaeffer-Fulton endospore method:***

a- Malachite green, the primary stain, is applied to a heat-fixed smear and heated to steaming for about 5 minutes. The heat helps the stain penetrate the endospore wall.

b- Remove slides from hot plate, cool, and wash under running tap water for about 30 seconds to remove the malachite green from all of the cells parts except the endospores.

c- Next, safranin, a counterstain, is applied for 30 seconds to the smear to stain portions of the cell other than endospores.

d- Wash with tap water and blot dry with bibulous paper and examine under oil immersion. In a properly prepared smear, the endospores appear green within red or pink cells.



3- Flagella Staining

Bacterial flagella are fine, threadlike organelles of locomotion that are so slender (about 10 to 30 nm in diameter) they can only be seen directly using the electron microscope. To observe them, the thickness of flagella is increased by coating them with **mordants** like tannic acid and potassium alum, and they are stained with pararosaniline (Leifson method) or basic fuchsin (Gray method).