

## DIFFERENTIAL STAINING, Part I

**Differential staining** is a procedure that takes advantage of differences in the physical and chemical properties of different groups of bacteria. It allows us to differentiate between different kinds of bacterial cells or different parts of a bacterial cell.

### I. GRAM STAIN

The most commonly used differential stain is the Gram stain, first described in 1884 by Christian Gram, a Danish physician. The Gram reaction divides bacteria into two groups, those which are Gram-positive and those which are Gram-negative. Those organisms which retain the primary stain (crystal violet) are stained purple and are designated Gram-positive; those which lose the crystal violet and are subsequently stained by a safranin counterstain appear red and are designated Gram-negative.

The conventional Gram-stain technique is described in the **Procedure** part of this handout; however, it is important to recognize early on that two aspects of the procedure are crucial:

1. The crystal violet treatment **must** precede iodine treatment. Iodine acts as a mordant, i.e., it increases the affinity of the cells for the crystal violet. Iodine alone has no bacterial staining capabilities.
2. Decolorization must be short and precise. Too long an exposure to 95% alcohol will decolorize Gram-positive as well as Gram-negative cells.

The Gram stain has been used as a taxonomic tool for many years, aiding in the classification and identification of bacterial cells. However, it is also useful in a broader sense, as there appears to be a close correlation between the Gram reaction and many other morphological and physiological characteristics of bacterial cells. For example, most of the rod-shaped, aerobic, spore-forming bacteria which are isolated from soil show a Gram-positive reaction, whereas most of the rod-shaped (bacillus-shaped) organisms which can ferment lactose and are common in the

intestinal tract of humans demonstrate a Gram-negative reaction. In addition, deadly proteinaceous exotoxins are typically synthesized by Gram-positive pathogens, whereas production of endotoxins seem to be a characteristic of Gram-negative cells.

The cell walls of the two groups are morphologically and chemically quite different. One explanation for the differential staining reaction emphasizes the higher lipid content of the cell walls in Gram-negative bacteria. During the decolorization step, alcohol may extract the lipids, increasing the porosity or permeability of the cell walls. Thus, the crystal violet-iodine complex is easily lost. The Gram-positive bacteria, however, do not have lipid-rich cell walls. Their cell walls become dehydrated during the alcohol treatment, decreasing the porosity so that the crystal violet-iodine complex is retained.

It is important to note that Gram-positive organisms are not always constant in their staining reaction. Older cultures of some Gram-positive bacteria are subject to autolysis. Enzymatic breakdown of the cell wall causes older cells to become Gram-variable (both red and purple cells present) or Gram-negative. The pH of the culture medium will also influence the staining of Gram-positive cells. Cultures for Gram staining should be grown in media low in sugars to avoid the formation of acidic end products during cell growth.

### **Material:**

1. Both plate and broth cultures (24-48 hour) of *Staphylococcus aureus*, *Bacillus megaterium* or *B. subtilis*, and *Escherichia coli* (try out plates first, then move to broth cultures as they are more difficult).
2. Gram stain reagents –
  - a. Crystal Violet
  - b. Gram's (or Lugol's) iodine solution
  - c. 95% Alcohol
  - d. Safranin
3. Tooth picks – Time permitting these can be used to scrape teeth and cheeks for Gram staining.

### **Procedure:**

- 1.** Prepare smears of the organisms listed above and heat-fix smear in the usual way.
- 2.** Allow the slide to cool, then flood with crystal violet. After one minute, pour off the stain and wash the slide gently but thoroughly with tap water.
- 3.** Flood the smear with Gram's (or Lugol's) iodine. After one minute, pour off the iodine solution and rinse with tap water. At this point, try to remove most of the excess water.
- 4.** Decolorize with 95% alcohol. Hold the slide at an angle and "drip" the alcohol over the smear. Observe these drips. As soon as the drips lose the faint touch of blue, rinse the slide with tap water **immediately**. The decolorization step should not have taken more than 15-20 seconds.
- 5.** Counterstain with safranin for 1 or 2 minutes and wash gently with tap water.
- 6.** Blot dry and examine the smear under oil immersion. Record results including sizes of each morphotype in microns.

## DIFFERENTIAL STAINING, Part II

With special techniques, it is possible to visualize endospores, capsules, cell walls, flagella, metachromatic granules and other intracellular constituents of microbial cells. The cellular component to be studied in this exercise is the endospore.

### II. SPORE STAIN

Endospores are specialized structures produced by two bacterial genera, *Bacillus* and *Clostridium*. Endospores (so called because the spore is formed **within** the cell) are easily seen with a phase microscope as highly refractile bodies. However, these structures are impervious to most ordinary stains, and are only occasionally seen as unstained areas in cells which have been stained with crystal violet or safranin. Because of this impermeability to **aniline dyes**, special staining techniques are used to differentiate between the endospore and the vegetative cell. Malachite green is placed on a bacterial smear and heat is used to drive the dye into the spores. The excess dye is rinsed off, and the unstained vegetative cells are counterstained with an appropriate dye of a different color, such as safranin.

#### Material:

1. Cultures (1-2 day old) of *Bacillus sp.*, e.g., *B. subtilis*, *B. megaterium*, and *B. cereus*. Dilute on slides with sterile saline solution when needed.
2. Malachite green
3. Safranin
4. Beaker of boiling water

#### Procedure:

1. Prepare a relatively thick smear of *Bacillus* (endospores may be few in number) and heat-fix the smear in the usual way.
2. Place the slide on a staining rack above a boiling water bath. Cover the smear with a small piece of paper towel or blotting paper, and saturate with malachite green.

3. Steam the slide for at least 5 minutes, replacing any liquid that evaporates with fresh malachite green. **DO NOT ALLOW** the smear to become dry and do not handle the hot slide with your fingers.
4. Allow the slide to cool to room temperature, then rinse with tap water. Shake off excess water.
5. Counterstain the smear with safranin for about 2 minutes. Rinse the slide with tap water and blot dry.
6. Examine the smear under oil immersion. Spores will be green and vegetative cells will be pink or red.

### III. CAPSULE STAIN

A capsule is a thick layer of complex polysaccharides (and in some cases, protein) firmly attached to the outside of the cell wall or outer membrane. Capsules are fragile; they are difficult to stain; and they are easily disrupted by heat. Their heat lability creates a problem: if you heat-fix the smear, you will destroy the capsule. Capsules can be observed, however, by negative stains or by combining a negative stain and a simple stain. “*Negative stains*” such as India ink, nigrosin and Congo Red stain the background but not the bacterial cells or capsules. The cells and the capsules that surround the cells would appear colorless against a dark background. The simple stain will then pass through the capsule and stain the cell.

#### Material:

1. 24-h nutrient broth culture of *Rhizobium sp.* and *Klebsiella sp.*
2. Congo Red solution
3. Maneval’s solution

#### Procedure:

1. Place a drop of Congo Red solution on a clean slide.

2. Mix one or two loops of one of the cultures into the drop of Congo Red solution. Each lab partner will do one culture.
3. Allow the smear to **air-dry**. **NOTE: Do not heat fix the smear.**  
**ADDED NOTE:** Make sure to do these steps right away at the beginning of class to minimize the waiting required.
4. Cover the smear with Maneval's solution and incubate for at least 1 minute.  
**CAUTION:** Maneval's solution contains phenol and is toxic. Avoid skin contact. Use of gloves might be a good idea here!
5. Rinse the smear gently with tap water and *carefully* blot dry.
6. Examine the smear under oil immersion.