GSt Gram Staining

Learning Objectives

The student will

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Use the bright field light microscope to view microbes under oil immersion, make accurate observations and appropriate interpretations and store the microscope according to lab procedures.
- Properly prepare a bacterial smear for accurate staining and describe the chemical basis for simple staining and negative staining.

Background/Theory

Differential staining distinguishes organisms based on their interactions with multiple stains. In other words, two organisms 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. This link to the OpenStax Microbiology text provides more detail on these differential staining techniques. (OpenStax CNX, 2018)

The **Gram stain** is a **differential staining** procedure that involves multiple steps. It was developed by Danish microbiologist Hans Christian Gram in 1884 as an effective method to distinguish between bacteria containing the two most common types of cell walls. (OpenStax CNX, 2018) One type consists of an inner plasma membrane and a thick outer layer of peptidoglycan. The other type consists of a double phospholipid bilayer with a thin layer of peptidoglycan between the two. The Gram Staining technique remains one of the most frequently used staining techniques.

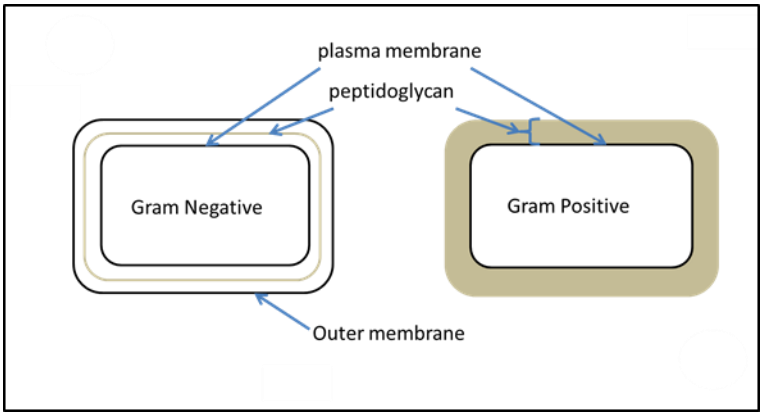

Figure 1 Simplified structures of Gram negative cells (left) and Gram positive cells (right)

The steps of the Gram stain procedure are listed below and illustrated in Figure. (OpenStax CNX, 2018)

1. First, **crystal violet**, the **primary stain**, is applied to a heat-fixed smear, giving all of the cells a purple color. You will recall that crystal violet is a basic stain (excess OH- ions). It adheres to the cell because the positively charged chromogen is attracted to the negatively charged cell as described in the Simple Staining exercise. (See figures 2 and 3.) This step is chemically

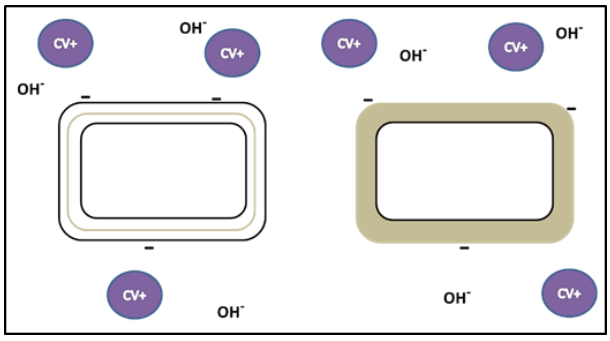

Figure 2 Crystal violet, a simple basic stain, is added.

identical to simple staining. (As far as I know, crystal violet is the only dye that can be used as the primary stain. I have seen, on unverified websites, methylene blue mentioned as a substitute for crystal violet. I have tried it and it does not work.)

2. Next, **Gram's iodine**, a **mordant**, is added. A mordant is a substance used to set or stabilize stains or dyes; in this case, Gram's iodine acts like a trapping agent that complexes with the crystal violet, making the crystal violet–iodine complex clump and stay contained in thick layers of peptidoglycan in the cell walls. All the cells become a deep purple color after this step. (See figure 4.)
3. Next, a **decolorizing agent** is added, usually ethanol or an acetone/ethanol solution. Cells that have thick peptidoglycan layers in their cell walls are much less affected by the decolorizing agent; they generally retain the crystal violet dye and remain purple. These are termed **Gram positive**. In the cells with the thin layer of peptidoglycan, the decolorizing agent easily washes the dye out of cells leaving them colorless. Cells that do not retain the CV-I are called **Gram negative**. Because the reagent reacts differently depending on the cell wall, this step makes Gram staining **differential** and is the most crucial. (See figure 5.)
4. Finally, a secondary **counterstain**, usually **safranin**, is added. This stains the decolorized cells pink. (OpenStax CNX, 2018) Like the primary stain, the counterstain step is chemically a simple stain. Its purpose is to make the colorless cells visible. The safranin chromogen actually adheres to all the cells. Because the Gram positive cells are retaining the dark purple CV-I complex, the pink safranin only shows up in the colorless cells. (See figure 6.)

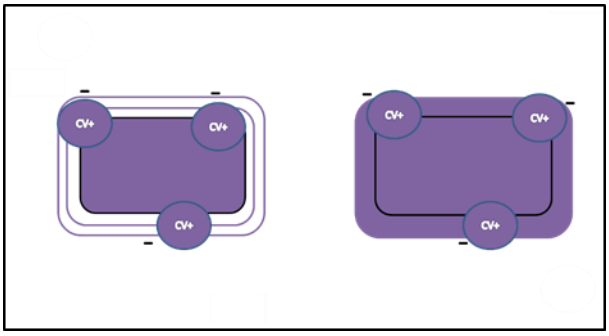

Figure 3 After crystal violet is added, all cells are purple.

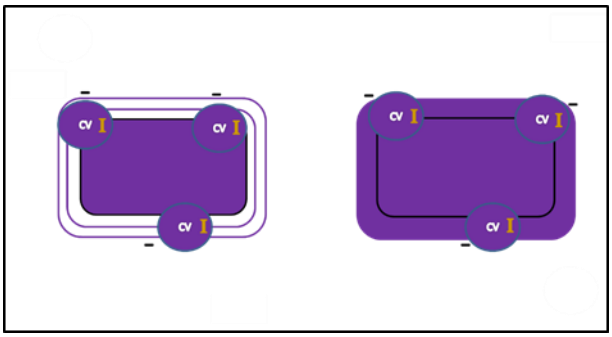

Figure 5 Iodine, the mordant, intensifies the color.

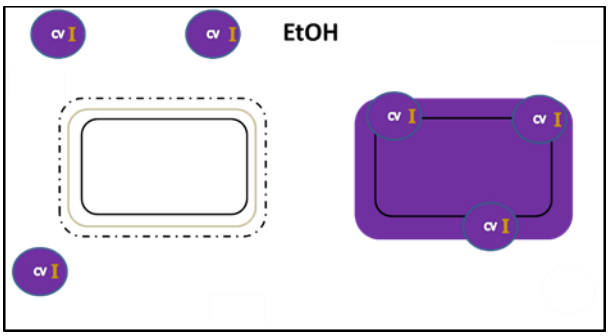

Figure 4 After decolorizing, Gram negative cells are colorless and Gram positive cells remain purple.

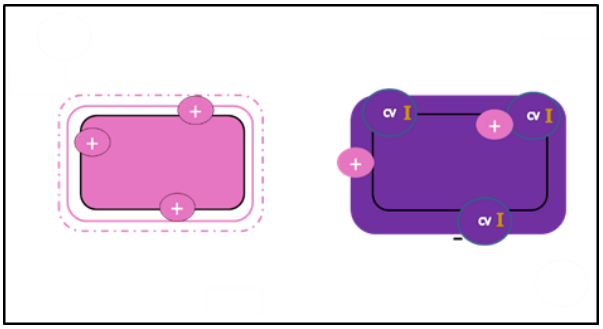

Figure 6 The counterstain imparts color to the colorless cells as a simple basic stain.

There are several important considerations in interpreting the results of a Gram stain. First, older bacterial cells may have damage to their cell walls. This is accentuated in Gram positive cells because the thick peptidoglycan begins to break down and

cannot retain the CV-I complex as efficiently. The result? Gram positive cells appear pink and may be misidentified as Gram-negative. Thus, it is essential to use fresh (24 hour) bacterial cultures for Gram staining.

Second, errors, especially decolorizing too long, can affect the results. (OpenStax CNX, 2018) If exposed to the decolorizer too long, the CV-I complex can be removed from Gram positive cells along with Gram negative cells making all types appear pink at the end. Conversely, if the decolorizer is not applied long enough, the CV-I complex will remain in the Gram negative cells and they will appear purple and can be misidentified as Gram positive. This is the most common mistake students make in Gram staining. Always do a control smear when Gram staining an unknown culture to verify that your technique is correct. Getting the decolorizing step correct is tricky and takes some practice. (OpenStax CNX, 2018)

The third cause of inaccurate Gram staining is a thick smear. If cells are clumped together on the slide, the cells will not make contact with each of the reagents equally. Some individual cells may not be decolorized enough and remain purple when other identical cells become decolorized. This will result in some cells in a pure culture smear appearing different from others. (OpenStax CNX, 2018)

Besides their differing interactions with dyes and decolorizing agents, the chemical differences between Gram-positive and Gram-negative cells have other implications with clinical relevance. For example, Gram staining can help clinicians classify bacterial pathogens in a sample into categories associated with specific properties. Gram-negative bacteria tend to be more resistant to certain antibiotics than gram-positive bacteria. (OpenStax CNX, 2018)

Gram Stain Procedure

1. Place a heat fixed smear on the staining rack.
2. Flood the entire slide with crystal violet and allow to sit for one minute.
3. Gently rinse with distilled water.
4. Add Gram's iodine to the slide, discard it into the staining tray and again cover the entire slide with the iodine. Allow to sit for one minute. By discarding the first few drops of iodine, you ensure that the mordant is not diluted by excess water drops left on the slide from the previous rinse.
5. Gently rinse with distilled water.
6. Make sure your water bottle is ready nearby. Hold the slide at an angle, <u>horizontally</u>. Apply 95% EtOH to the slide until the stain stops coming off the slide and the decolorizer runs clear. This only takes 3-4 passes across the slide.
7. <u>Immediately</u>, rinse with distilled water, gently. This will stop the decolorizing process.
8. Apply the counterstain, safranin and allow it to sit for one minute.
9. Gently rinse with distilled water.
10. <u>Gently</u> blot in a fairly clean area of a bibulous paper book. Refrain from blotting after each rinse step. This is unnecessary and may result in the loss of cells from the slide making the few that are left more difficult to find.

In this exercise you will stain a slide with 2 smears. On one side of the slide you will place a mixed culture control smear containing *E. coli*, a known Gram negative rod and *Staphylococcus epidermidis*, a known Gram positive coccus. You will know you have stained the slide correctly if all the rods are pink and all the cocci are purple. Be sure you are looking for cell morphology and color. If you are not tuning into the details you may not interpret your results accurately. For example, in a cursory glance you may see both colors, but a closer look may reveal an unevenly stained slide with a clumped mass of cells (where you cannot distinguish individual cell morphology) retaining the purple color and individual cells, both rods and cocci, staining pink.

Once you know your technique produced accurate results, you will have confidence in the results on the second smear on the same slide. This smear will be a sample from the edge of your gums. This gum line scrape will contain numerous bacterial types along with some of your own epithelial cells. Since you are a eukaryote, these cells will be **HUGE** compared to the bacterial cells. Because you do not know what bacteria to expect, you will observe the control smear first to make sure your staining technique is accurate and then proceed to observing the Gum line smear.

Experiment/Exercise

Materials per student pair

4 Microscope slides
1 bottle Crystal violet stain
1 bottle Gram's iodine
1 bottle 95% Ethanol (EtOH)
1 bottle Safranin stain
Sterile toothpicks

Cultures

Fresh overnight broth mixed culture containing
E. coli and
Staphylococcus epidermidis

Procedure

1. Each person will make two identical slides.
   a. Divide each slide in half. Label one side MX and the other G.
   b. On the MX side, aseptically make a smear with the mixed culture control. Be sure to sterilize your loop after spreading cells on one slide and before getting more cells for the second slide. Be sure to make the smear about the size of a nickel.
   c. On the G side of each slide, aseptically make a gum line smear as follows. Place a loopful of water on the slide as you would if making a smear from a solid medium. With a sterile toothpick, scrape your teeth at the gum line gently. (Do not draw blood.) Spread the sample on the slide in the water. Allow both slides to air dry and then heat fix both slides. (See figure 7.)
   d. Set one slide aside as your back up.

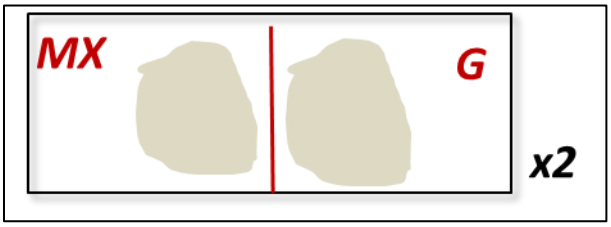

Figure 7 Each person makes 2 identical slides as described. MX is the mixed culture control smear and G is the gum line sample.

2. Gram stain one slide using the procedure above. Treat all parts of the slide the same.
3. Find the cells under oil immersion. As always, you will need to start with the scanning objective. Follow the steps described in Microscope Theory.
4. Evaluate your Gram staining technique by locating both rods and cocci. Determine if each is the correct color. Make sure that you are not just looking for both colors. You need to make sure that the rods are pink/red and the cocci are dark purple. Consult with your instructor. Note that on high power (high dry), the resolution is not good enough to determine the cell morphology and color. You must find cells under oil immersion to determine these characteristics.

5. If your staining technique is good, your instructor will initial your Data and Observations table and you can then go on to record your observations. Use one row of the table for *E. coli* and the next line to make observations for the *S. epidermidis*. Remember, the first time you write the organism name, write it out in full scientific form.
6. If you need to practice again, discuss with the instructor how you might improve and then repeat the Gram stain with your back-up slide.
7. Once you know your technique with this particular slide is accurate, observe the Gum smear on the same slide.
8. One of the data table rows may be used to observe the relatively gigantic eukaryotic cells present. These are your epithelial cells. Call this sample "Gum line, human epithelial cell"
9. Fill in 2 additional rows in the data table with 2 different groups of bacterial cells. Call these "Gum line, cell type #1", and "Gum line, cell type #2."
10. If you did not need your second slide, leave it with the instructor as an extra slide for another student who may need it. Dispose of any slide with immersion oil on it in the slide disposal container.
11. Empty stain in the staining tray into the stain disposal container, rinse the tray into the sink and leave upside-down next to the sink along with the rack.

GSt

Blank

Lab Report: GSt Gram Staining

Name _____
Lab Section _____

Data and Observations

Organism	color	Gram Rxn	Drawing	Morphology and Arrangement	instr initials
Mixed culture control Organism: _____ _____					
Mixed culture control Organism: _____ _____					
Gum line, human epithelial cell					N/A
Gum line, cell type #1					N/A
Gum line, cell type #2					N/A

Post Lab Questions

1. Explain the major differences between the Gram positive and the Gram negative cell wall.

2. What are the 4 major steps in Gram Staining and what is the role of each reagent?

3. A student divides a slide in half and places a mixed culture smear of *E. coli* and *S. epidermidis* on one side and a pure culture smear of an unknown microbe on the other. They Gram stain the slide and begin their observations by looking at the mixed smear under oil immersion. Evaluate the student's Gram staining technique when the following results are observed. Explain what the student may have done wrong. If they also observe the unknown smear on the same slide, are their results likely to be accurate?

    a. Both the rods and the cocci appear purple.

    b. You can only find rods and they are all pink.

4. What is wrong with putting an unknown organism and the control mixed culture smear on <u>different</u> slides?

5. Why would a health care provider be interested in knowing the Gram reaction of a pathogenic bacterium?

6. In the gum line smear, you viewed some of your epithelial cells. They appear extremely large compared to the bacteria. What component of a bacterial cell wall is lacking in eukaryotes?

   What color should your epithelial cells be? Briefly explain.

   What color did they actually appear?

7. Instead of the usual control organisms, you use *Bacillus cereus*, a Gram + rod, and *Moraxella catarrhalis*, a Gram – coccus. If stained correctly, what result do you expect to see?

8. If a student performs the perfect Gram stain except for the following mistakes, what result (color) would you expect to see if the cells were Gram negative? Gram positive?
   a. The student skipped the iodine step.

   b. The student left the safranin on for an extra 15 seconds.

   c. The student delayed rinsing the EtOH off the slide for a minute because they did not have enough water in their bottle and had to refill the bottle.

   d. Methylene blue was used instead of safranin.

   e. The student switched crystal violet and the safranin.

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References

OpenStax CNX. (2018, Mar 19). OpenStax Microbiology. Retrieved from http://cnx.org/contents/e42bd376-624b-4c0f-972f-e0c57998e765@4.24