Exercise 3: Performing the Gram stain

Goals:

- Practice and develop microscope skills in the interpretation of Gram stained smears
- Demonstrate and explain procedures for the Gram stain
- Interpret and evaluate Gram stained smears

Specific Objectives

- List the reagents and steps used in a Gram stain
- Explain the purpose of each reagent and step in the Gram stain
- Define the terms in bold
- Evaluate a Gram stained smear and troubleshoot Gram staining problems
- Describe the Gram stain reaction, cell size, cell shape, and arrangement of four common bacterial species
- Use the proper technique to operate the bright field microscope for locating and viewing organisms on a stained smear
- Interpret unknown slides for Gram stain reaction, cell size, cell shape, and arrangement
- Describe limitations of the Gram stain

Introduction

The Gram staining method, named for Danish bacteriologist Hans Christian Gram, is a **differential stain**. It is one of the most important staining techniques in microbiology and is almost always the first test performed for the identification of bacteria. The **Gram stain** differentiates between two major cell wall types. The microorganisms that are stained by Gram's method are commonly classified as **Gram positive** (purple) or **Gram negative** (pink.) The difference in the staining properties of bacterial cells reflects the difference in cell wall composition**,** most importantly, the amount of peptidoglycan present. **Peptidoglycan,** found only in bacteria, is a high molecular weight repeating carbohydrate polymer linked by amino acid bridges, which forms the structural backbone of the bacterial cell wall.

The Gram positive cell wall consists of a thick sheath of **peptidoglycan** with tightly bound acidic polysaccharides, including teichoic acid and lipoteichoic acid. Gram positive bacteria are those bacterial species with cell walls containing relatively large amounts of peptidoglycan and no **lipopolysaccharide**.

The **Gram negative** cell wall consists of an **outer membrane** containing lipopolysaccharide (**LPS**), a thin shell of peptidoglycan, periplasmic space, and an **inner membrane**. Gram negative bacteria are those bacterial species with cell walls containing lipopolysaccharide and small amounts of peptidoglycan. The cell wall for Gram negative microorganisms has a higher lipid content compared to Gram positive cells. Gram negative bacteria do not retain crystal violet. Rather, these stain pink or red following decolorization with alcohol and subsequent application of safranin, the counterstain. **Gram variable** refers to Gram positive cells that sometimes stain Gram negative, as seen with clinical samples.

Patient samples that are smeared and Gram stained are referred to as **Primary Gram Smears (PGS).** This initial step guides identification and work up of the sample. The smear from a patient source, will often appear very thick due to the extra protein present in the form of mucus and host cells. The method of transferring patient sample material to a glass slide will vary greatly depending on the sample type and is beyond the scope of this exercise. If you are interested, you may refer to Appendix P, Section 3 for details.

Components of the Gram stain:

The **primary stain**, crystal violet, enters both Gram positive and Gram negative cells, staining them deep purple. This basic dye diffuses throughout the bacterium and is held in both the bound and unbound states. The **mordant,** Gram's Iodine, is added next. A **mordant** makes the staining solution stain more intensely. Wherever the mordant meets the basic dye, a water insoluble lake is formed and is composed of a stable crystal violet-iodine complex (CV-I) within the cell. The lake is only moderately soluble in low molecular weight alcohols and acetone. Next is **decolorization,** which is the treatment of the stained cells with alcohol or acetone. This step removes the lipids in the walls of the Gram negative bacteria causing them to become porous. The CV-I complex leaks from the cells due to the increased permeability, and the cells become colorless. These colorless cells take up the safranin (or counterstain) and appear pink or red. These cells are called Gram negative. The high peptidoglycan content in Gram positive cell walls responds to the alcohol or acetone treatment by shrinking. This traps the CV-I complex in the Gram positive cells causing these cells to remain purple even after **counterstaining** with safranin.

Table 1: Components of the Gram stain

Reagent	**Reagent color**	**Function**	**Cell color after application**
Crystal violet	purple	Primary stain	All cells purple
Gram's Iodine	Yellow/orange	Mordant Forms complex with Crystal Violet	All cells purple
Acetone or Ethanol	colorless	Decolorizer Dissolves lipid in the outer membrane of Gram negative cells Dehydrates the cell wall of Gram positives, "trapping the stain"	Gram positives purple Gram negatives colorless
Safranin	red	Counterstain Stains decolorized bacteria	Gram positives purple Gram negatives pink

Observing and Evaluating Gram Stained Smears

A Gram stained smear should appear only lightly colored to the naked eye. A good slide is evenly stained and the bacteria are spread thinly enough that you can identify individual cells. The bacteria should not be in clumps, as this will alter the amount of stain retained in that area. The 10X (low power) objective should be

used to focus in on a region of the smear. Remember to use your **coarse adjustment** first, then your **fine adjustment** to focus on your specimen. You may choose to view the smear at 40X (high power). Use only the fine adjustment at this magnification. Now you should be able to see cells, but probably cannot make out their shape. Moving to 100X will allow you to completely evaluate the cells on your smear. If you feel comfortable using your scope, you may wish to skip from 10X straight to 100X (oil immersion) to observe the bacterial cells. Recall that oil is necessary when viewing specimens with the 100X lens to increase **resolution**. These colors are hard to differentiate at first and you should find an area of your slide in which the cells are not too densely packed in order to observe them. Once stained, bacterial cells can be observed for Gram stain reaction, size, **cellular morphology**, and spatial **arrangement**. Arrangements of cells are best observed from broth cultures because the emulsification process disrupts the natural arrangement from colonies "picked" from solid media.

Note: Some individuals who are Red/Green colorblind may find it difficult to perceive the pink/red appearance of Gram negative cells. In this case, Bismarck Brown may be substituted for Safranin as a counterstain. With Bismarck Brown, Gram negative cells will appear a very light brown.

Limitations and common pitfalls of the Gram Stain

It is important to remember the following things when preparing Gram stains. As Gram positive cultures age, the cell walls tend to become naturally more porous, allowing the CV-I complex to be extracted with alcohol or acetone. This can cause them to appear red when counterstained. To avoid this problem, always use young cultures (16 to 24 hours) to obtain accurate Gram stain results. Gram positive cells grown under acidic conditions can also lose their ability to retain the CV-I complex, resulting in erroneous Gram reactions. To avoid this problem, use cells grown in a neutral medium for Gram staining. For proper decolorization, the smear should be a thin, uniform film. Thick smears will make it very difficult to discern useful information about the cells.

Decolorization is the most common place for error in the Gram stain procedure. It is easy to overdecolorize a slide. When a slide is **overdecolorized**, cells that are normally Gram positive will appear Gram negative. This results in what is called a false Gram negative. Students may overcompensate and, as a result, they may underdecolorize their smears. When **underdecolorization** occurs, cells that are normally Gram negative will appear Gram positive resulting in a **false Gram positive**.

While the Gram stain is differential only for bacteria, some yeasts and fungi may retain the color of the crystal violet and are often referred to as Gram positive. *Candida albicans*, for example, is sometimes misinterpreted as a Gram positive coccus. Animal cells cannot retain the CV-I complex but retain the counterstain, so they *appear* uniformly Gram negative when viewed microscopically. For this reason, evaluation of clinical specimens requires a great deal of practice; it can be difficult to identify bacterial cells among the background of pink-stained host cells and mucus.

Materials

Prepared and heat fixed smears from last lab

- *S. aureus* and *E. coli* broth smears
- *B. cereus* and *P. aeruginosa* plate smears

Gram staining supplies

Procedure

Part 1: Stain your bacterial smears using the Gram's stain method

1. Place the fixed smear on the staining rack over the sink
2. Cover the smear area with Gram's Crystal Violet and leave on for 30 seconds. Rinse the slide with a gentle stream of water
3. Cover the smear with Gram's Iodine and leave on for 30 seconds. Rinse the slide with a gentle stream of water
4. Next, apply a gentle stream of Gram's Decolorizer to the slide for 5 to 15 seconds. You should see purple stain dribbling off the end of the slide. Stop applying decolorizer as soon as the runoff is no longer purple. Rinse the slide with a gentle stream of water
5. Cover the smear with Gram's Safranin for 30 seconds, and then rinse with water
6. Allow the smear to air dry

Part 2: Visualize your bacterial smears

For bacterial specimens you will need to examine your smear using the 100X objective lens.

1. Remove your microscope from your cabinet. Grasp the microscope with one hand on the base and one hand on the carrying arm
2. Remove the dust cover and store in your cabinet
3. Plug in your microscope
4. Turn on the light and adjust the brightness until you are comfortable
5. Adjust the ocular lenses until only one circle of light is visible
6. Raise the sub-stage condenser
7. Rotate the nosepiece to get the 10X (low power) objective into position over the stage
8. Place the sample slide on the stage using the slide holder to grip it
9. Use the coarse (larger) focus adjustment knob to focus on your specimen
10. While looking through both eyepieces, first adjust the coarse focus adjustment knob until the subject comes into view. Then, adjust the fine focus to get a clear image. **Note: Do not readjust the coarse adjustment knob once you have your sample in focus.**
11. Adjust the iris diaphragm lever to reduce or increase the light intensity. When increasing the lighting, be sure to open the diaphragm first instead of increasing voltage on your lamp. (This will greatly extend the life of the lamp).
12. Once an image is visible, move the slide around by turning the knobs that move the mechanical stage
13. For increased magnification, swing the 40X (high dry) objective into position. Readjust focus, if necessary, using the fine adjustment knob only
14. For greatest magnification, swing the 40X objective out of position, and add a drop of immersion oil to the slide. Swing the 100X (oil immersion) objective into position. It should touch the drop of oil as it sets into position. The oil is necessary to permit the maximal amount of light into the lens. Adjust the fine focus and iris diaphragm as necessary. Never use the coarse adjustment knob with oil immersion.
15. If you lose focus, do not move the 40X objective back into place. Oil will permanently damage the lens. Go back to the 10X and refocus. Once in focus, swing the 100X objective into place. Ask for help if needed.
16. Record your observations in the table below.

Part 3: Storing your microscope

Recall that the microscopes are used by multiple people throughout the semester. Clean and store your microscope properly to keep it in good working order for yourself and your colleagues.

1. Remove the slide from the stage. Slides that will not be needed again can be disposed of in the appropriate containers. Slides to be kept should be placed in a labeled slide box.
2. If immersion oil has been used, blot any spills on the stage and gently blot the 100X objective using a kimwipe.
3. Rotate the low-power (4X) objective into position.
4. Neatly, wrap the electrical cord around the base. Replace the dust cover.
5. Return the microscope to its correct place in the cabinet.

Observations

Organism	Gram reaction	Size	Morphology	Cellular arrangement
Escherichia coli				
Staphylococcus aureus				
Pseudomonas aeruginosa				
Bacillus cereus				

References

Tessmann, B. (2019). *Introductory Microbiology Manual.* Unpublished Manuscript.

Study Questions

1. List the steps of the Gram stain in their proper order.

2. What part of the bacterial cell is most involved with Gram staining and why?

3. What step is most likely to cause poor results in the Gram stain and why?

4. In a clinical specimen, why would host cells appear Gram negative?

5. Explain how improper Gram staining can result in a false Gram negative.