

Cytological Stains: Gram, Acid-fast, and Spore Stains

INTRODUCTION

In microbiology, organisms are so small that additional techniques are often required for proper viewing under the microscope. **Cytological stains**, or dyes that stain cells or cellular features, are useful in that they enhance the contrast in a microscopic image by highlighting certain cells or structures of cells. In this lab we have the opportunity of performing simple, gram, acid-fast, and the spore stain.

A **simple stain**, in which only one stain is involved, is the simplest form of staining a cell. Simple staining usually highlights only one feature of the cell or only one type of cell, while additional steps involving counterstains can be used to highlight other features in more complex staining techniques. In this lab, we are using the simple stain “crystal violet” to color all bacterial cells in order to better observe bacterial cellular **morphology** and **arrangement**.

Gram Stain

Gram staining is a multiple staining procedure that involves the use of several dyes to differentiate bacteria into two basic groups based on the composition of their cell walls: Gram positive and Gram negative bacteria. Gram staining, unlike simple staining, is a **differential** stain in that it renders one type of bacteria one color and other types of bacteria another color. Gram staining uses the primary dye crystal violet to stain the cell walls, iodine as a **mordant** (dye fixative), gram decolorizer as a challenge rinse, and finally safranin to counterstain cells. The resultant color of the bacteria is based on the composition of their cell wall in that gram negative bacteria show up red/pink while gram positive bacteria end up purple/blue (Figure 1). The resulting purple/blue gram positive bacteria is due to the thick peptidoglycan layer within the bacterial cell wall, where the pink/red color of gram-negative comes from the lack of a thick peptidoglycan layer with rather a larger lipopolysaccharide layer found in gram-negatives (Figure 2). In other

words, Gram negative cells have mainly a lipid cell wall that allows the challenge rinse to penetrate the cell and rinse out the crystal violet-iodine complex, rendering the gram negative cells colorless. In order to see the Gram negative bacteria, they must be counterstained with safranin. On the other hand, Gram positive bacteria have a thick peptidoglycan layer in the cell walls that becomes dehydrated and tightens after being rinsed with ethanol or acetone in the rinsing process. This tightening of the cell wall does not allow the rinse to enter the cells, therefore leaving the primary crystal violet dye intact. The gram positive bacteria remain purple/blue since even when the counterstain is applied; the counterstain does not change the dark purple color.

A successful gram staining procedure relies upon several factors: the integrity of the cell wall, age of cells, and proper preparation of the slide. Older and damaged cells do not retain the dyes properly and allow the challenge rinse to penetrate regardless of gram status. Also, bacteria that have been overheated during the fixation process can have breached cell walls that allow the rinse to penetrate, resulting in skewed results. It is imperative to carefully gram stain young, reproductive cells.

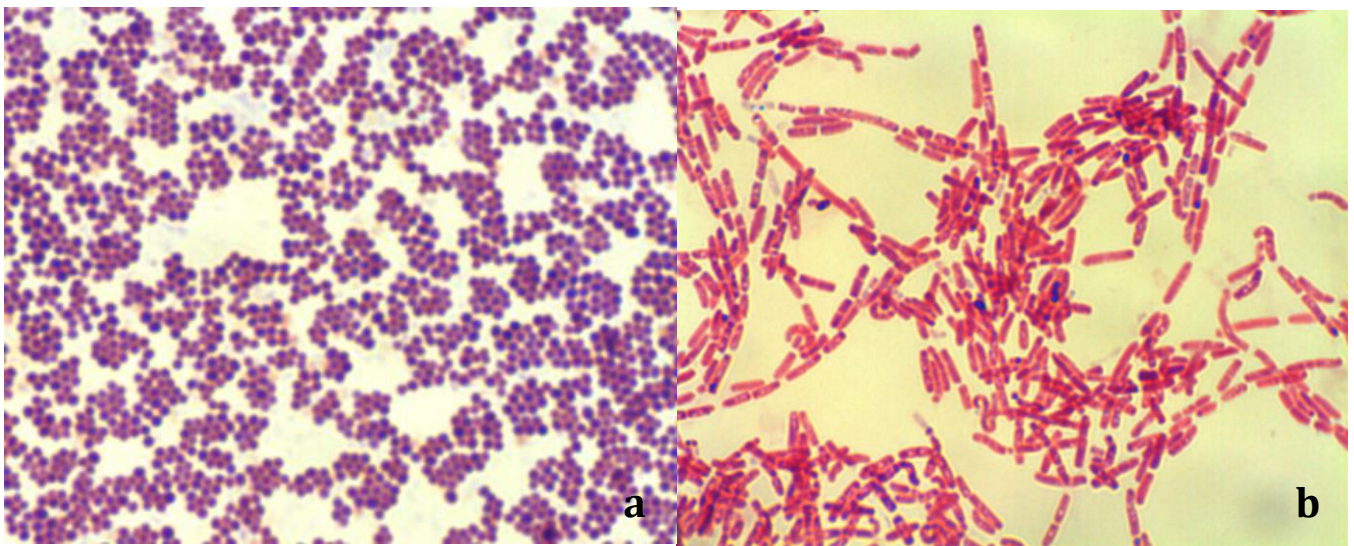


Figure 1. Gram staining of two bacteria types a) Gram Positive Cocci in clusters (Staph) b) Gram Negative Bacillus in chains (Strep)

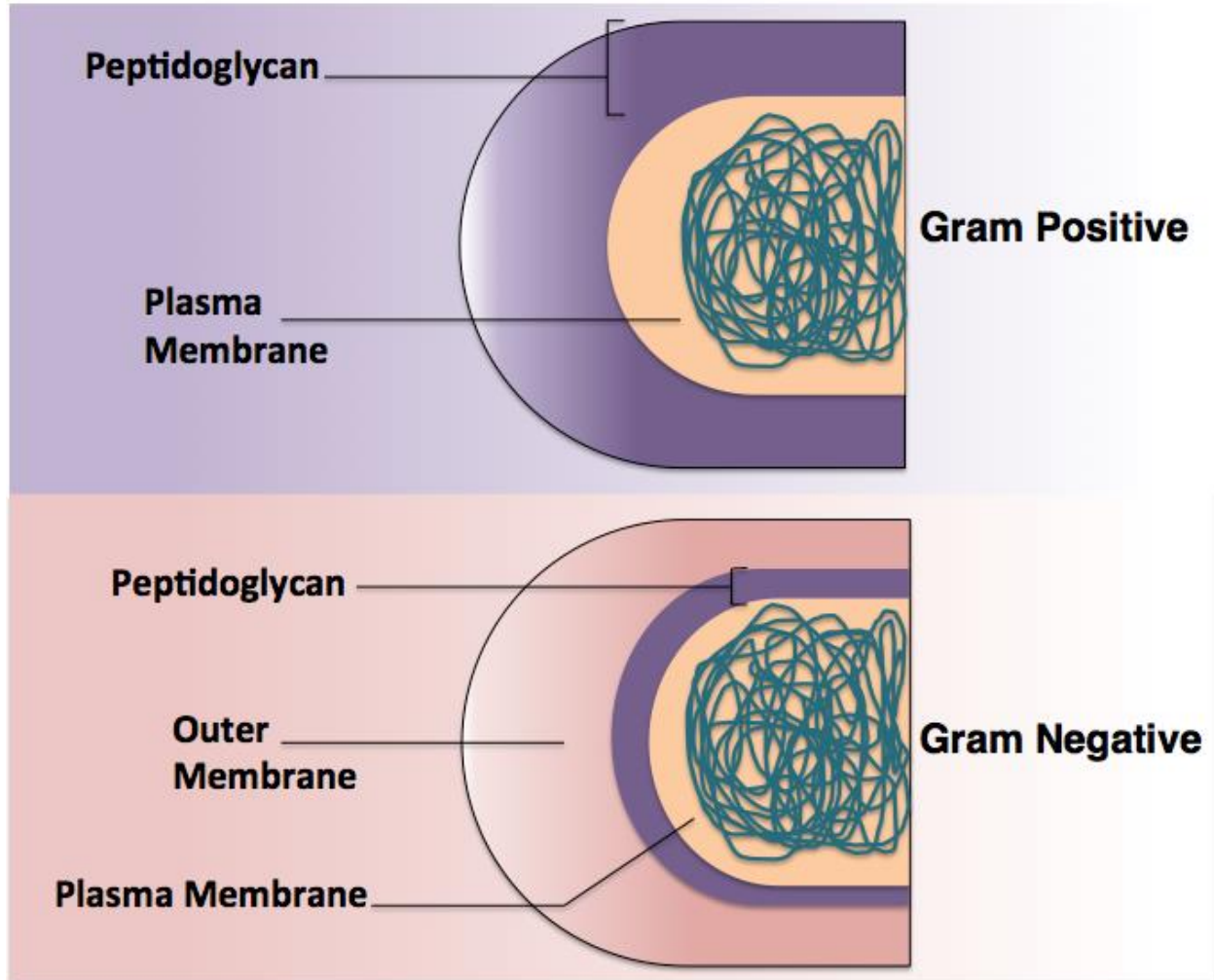


Figure 2. Diagram of the cell structure of Gram negative and Gram positive bacteria.

Acid Fast Stain

Acid-fast bacteria are Gram positive bacteria that possess mycolic acid or waxes in their cell walls. Acid fastness is a physical property of bacteria that is usually characterized as resistance to decoloration by acids in laboratory staining procedures. In order to properly stain acid-fast bacteria, a heat-driven stain is used in order to drive the stain through the cell wall. The acid-fast stain uses an acid alcohol (3% HCl in alcohol) challenge rinse in order to wash the primary stain from the cells that do not possess waxes in their cell walls. The acid-fast stain is an important stain in the medical field where quick detection of pathogenic bacteria is required such as for bacteria responsible for tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*).

Figure 3. Acid-Fast Stain showing

The Spore Stain

Another common differential cytological stain that is often used in the lab and in the medical field is the spore stain. The spore stain is a stain that differentiates between sporulating, or spore-forming bacteria, and non-spore forming bacteria and vegetative cells. **Vegetative cells** are bacterial cells that are actively growing, metabolizing, and dividing. When vegetative cells are exposed to environmental stress, especially nutrient deprivation, they begin to die. Some species of bacteria, however, are able to survive these harsh conditions including species from the *Bacillus* and *Clostridium* genus. Some bacteria from the *Bacillus* and *Clostridium* genus circumvent the consequences of environmental stress by forming endospores.

Endospores are clonal, dormant and metabolically inactive forms of the bacteria and contain the complete set of genetic material, ribosomes, and enzymes from the vegetative cell. Spores are highly resistant to heat, UV radiation, and chemicals and allow the bacteria to effectively survive harsh environmental conditions. Spores are resistant because they are comprised of a tough proteinaceous covering of keratin called the **spore coat** (Figure 4). Beneath the spore coat is the **spore cortex** that is made up of peptidoglycan. After the cortex lies the spore wall that surrounds the **core**, the central compartment for the DNA, ribosomes, and enzymes necessary for growth.

Once an endospore is formed, and once matured, the bacteria cell may lyse and release the endospore to become an **exospore**. Once the spores are exposed to a favorable environment, the endospore will develop and revert back to the vegetative cell in a process called **germination**.

Spores are refractive to most stains, where they do not absorb the dye but appear as clear areas in a Gram stain. In the spore stain heat must be used in order to melt the keratin spore coat to allow the Malachite Green spore dye to enter the cell. After decolorization with water, the cells lose the color from the malachite green while the spores retain the dye. The cells are then counterstained with Safranin to observe the vegetative cells. After a proper staining procedure, the vegetative cells should appear pink while the endospores and exospores appear blue/green (Figure 5).

Spore staining is an important aspect in the medical clinic where characterization of spore-forming pathogenic species of *Bacillus* and *Clostridium*. Species including *Clostridium perfringens*, *Clostridium botulinum*, and *Clostridium tetani* are the causative agents of gas gangrene, botulism, and tetanus in humans, respectively. Species such as *Bacillus anthracis* and *Bacillus cereus* are causative agents of anthrax and food poisoning.

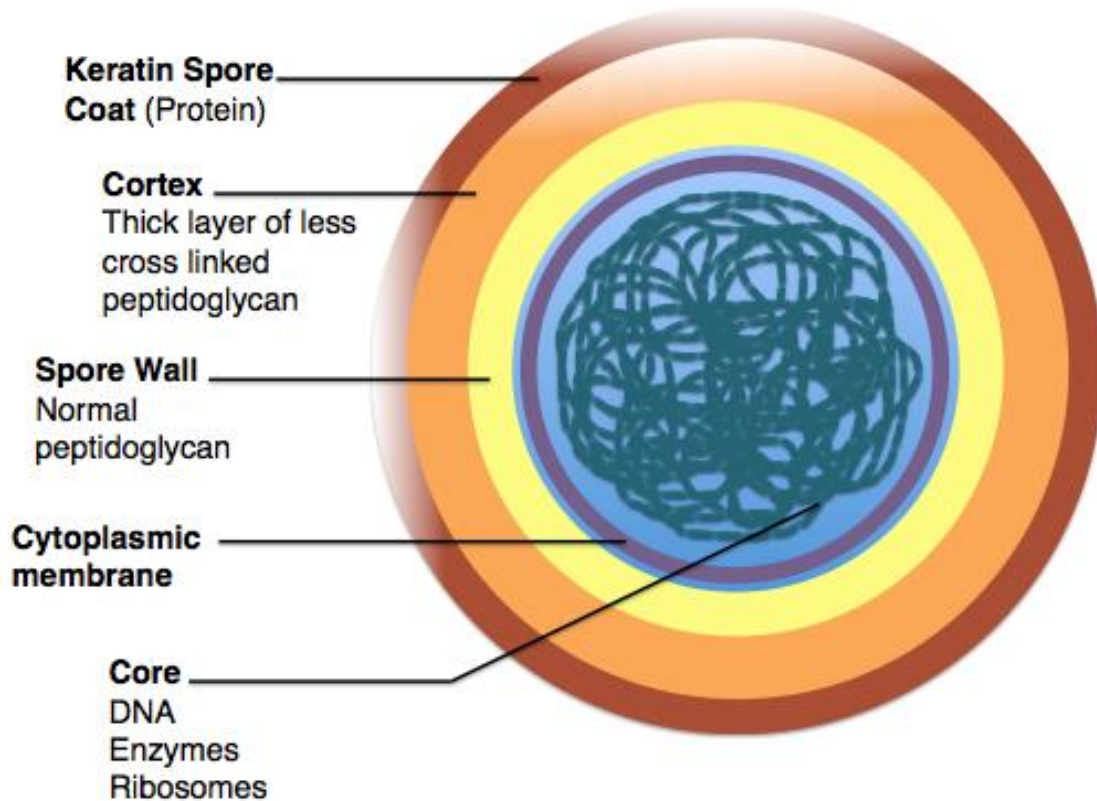


Figure 4. Diagram of the structure of a bacterial spore.

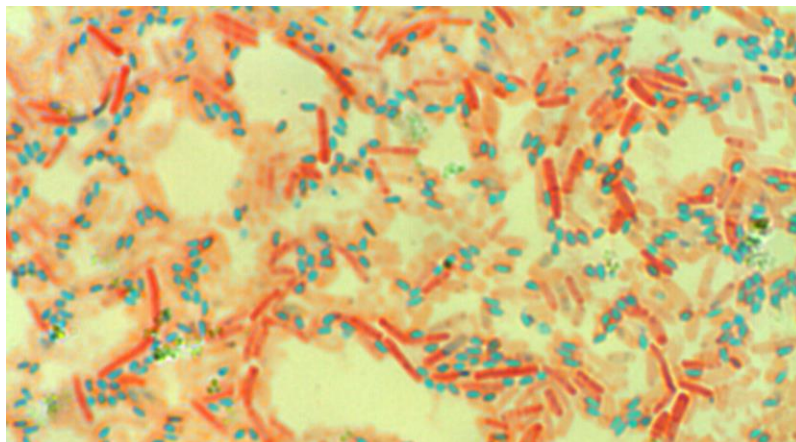


Figure 5. A Spore stain of a *Bacillus* strain with blue/green spores. Dark red cells indicate vegetative cells and lighter cells are dead/dying cells.



Part I. Simple Stain

MATERIALS PART I

1. (2) Slant cultures of chosen bacteria.
2. Inoculating loop
3. Glass slides

PROCEDURES PART 1

1. Prepare a slide with two smears of the two bacteria samples given. Refer to Appendix Figure 7 for heat fixed smear preparation.
2. Cover the smears with Crystal Violet for 1 minute.
3. Rinse with water and pat dry with paper towel.

Part II. Gram Stain

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MATERIALS PART II

1. **(2) Slant cultures of given bacteria.**
2. **Inoculating loop**
3. **Glass slides**
4. **Gram staining reagents**
5. **Toothpicks**
6. **Nutrient agar plate from experiment 1**

PROCEDURES PART II

1. Prepare FOUR separate smears of bacteria onto TWO slides. The two bacteria provided, the bacteria from Experiment 1, and the bacteria derived from the mouth.
2. For the mouth bacteria, choose one lab member to be source. Have them grab a toothpick and scrape between their teeth thoroughly.
3. Place the teeth scraping onto a water droplet on a slide and air dry.
4. Stain the smears with Crystal Violet for 1 minute, rinse with water.
5. Stain the smears with Gram's Iodine for 1 minute.
6. Carefully rinse off the blue color from the smears with Gram decolorizer or acetone.
7. Rinse with water.
8. Counterstain the cells with Safranin for 1 minute.
9. Rinse with water, and pat dry with paper towel.

Part III. Acid- Fast Stain



MATERIALS PART III

1. **Slant cultures of *Mycobacterium*, and *Staphylococcus aureus***
2. **Carbol Fuchsin**
3. **Acid Alcohol**
4. **Methylene Blue**

PROCEDURES PART III

1. Prepare ONE slide with a heat fixed smear of *S. aureus* and one of *Mycobacterium*
2. Cover the smears with a small piece of paper towel.
3. Flood the slide and paper towel with Carbol Fuchsin, and heat the slide over steam for 10 minutes. Do not allow slide to dry.
4. Cool the slide, remove paper, and then rinse with water.
5. Rinse the slide with acid alcohol until color does not run out.
6. Rinse with water
7. Counterstain the smears with Methylene Blue for 1 minute.
8. Rinse with water, and pat dry with paper towel and view at 1000X.

Part IV Spore Stain

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MATERIALS PART IV

1. Two Nutrient Agar Slants cultures of *Bacillus* or *Clostridium* species; One grown for 24 hours and the other for 48 hours.
2. Malachite Green Spore Stain
3. Safranin
4. Slides and Slide holders
5. Hot plate
6. Beaker with slide holder for steaming

PROCEDURES PART IV

1. Prepare ONE slide with a heat fixed smear of *Bacillus* at 24 hours and one of *Bacillus* at 48 hours onto one slide. Use a marker to label the back of the smear in order to know which smear is which.
2. Cover smears with a piece of paper towel.
3. Steam the slide for 10 minutes. Do not allow slide to dry by adding more dye dropwise.
4. Remove paper (biohazard) and allow slide to cool
5. Rinse with water
6. Counterstain slide with Safranin for 1 minute
7. Rinse with water, pat dry using paper towel, and view at 1000X.

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Procedures for Next Lab

MATERIALS

- 1. Two Sabouraud dextrose agar plates**
- 2. Facility of preference (indoor and outdoor)**
- 3. Parafilm**
- 4. Marker**
- 5. Sandwich bags**

PROCEDURES

1. Obtain 2 Sabouraud dextrose agar plates and have one member of the group take it home. Make sure it is sealed with parafilm.
2. Store the plates in the fridge until exposure time. Make sure to expose the plates a **5 days** before lab.
3. Take one of the Sabouraud dextrose agar plates and remove the lid and place it in the indoors of the chosen facility. Expose the plate for 15 minutes.
4. Take the other petri plate, with the lid removed; place the other plate on the outdoors of the chosen facility.
5. After 15 minutes of exposure, place the lids back onto the plates, and seal the dish with a piece of parafilm and place in ziplock bag and keep at room temperature.
6. Bring the plates into lab the on the next lab day.

Part I Microscopic Observations (5 points)

Directions: Complete the chart below by including sketches/details of your microscopic observations.

Include color, morphology and arrangement in characteristics. Indicate NA (if not applicable).

Organism	Stain technique	Sketch	Gram Reaction	Characteristics	Total Mag
Finger Bacteria					
Mouth Bacteria					

Part II. Questions: Gram Stain

Directions: Answer the following questions using full sentences. **(5 points)**

1. Explain the difference in the cell walls of the organism Gram stained based on the results, including why the cells are blue/purple or pink/red at the end of the process?

2. Are there any differences between the bacteria found on the skin and the bacteria found in the mouth? Why do you expect any differences?

3. Predict the results when making the following errors in gram staining:
 - a. Failure to decolorize

 - b. Failure to apply Safranin

 - c. Gram Staining old cells

 - d. Forgetting to heat fix

Part III. Questions: Acid Fast Stain

Directions: Answer the following questions using full sentences. **(5 points)**

1. What substance is found in the cell walls of Acid-fast microbes? Which of the two bacteria stained contained the substance?
2. What would be the consequences of forgetting to use heat in the acid-fast stain?
3. Would acid-fast bacteria have any advantages over non-acid fast bacteria?
4. Which bacteria do you expect to be more resistant to chemical agents, acid fast or non acid-fast bacteria? Why?
5. In the Gram Stain, the mordant used to fix the dye within the cells was Gram's Iodine. What is the mordant in the case of the acid-fast stain? (Mordant= Fixative, stabilizer, maintains, preserves)

Part IV. Questions: Spore Stain

Directions: Answer the following questions using full sentences. **(5 points)**

1. Why are spores so difficult to stain using common staining methods?
2. What differences can you observe between the two smears of *Bacillus* 24 after hours and *Bacillus* after 48 hours? Include differences in cell morphology, color of vegetative cells, relative number of spores, and any other obvious differences.
3. What will be the consequences of forgetting to use heat in the Spore Stain?
4. In a clinical setting, what advantages do sporulating bacteria have over non-sporulating bacteria?
5. Why do you suppose most disinfectants require that you leave the surface covered with a disinfectant for a certain amount of time?

