

BACTERIAL CYTOLOGY

In the previous two exercises you learned techniques for aseptic manipulation and microscopic observation of microorganisms. In this week's exercise you will employ both skills to study some of the microscopic features of bacterial cells. You will learn how to prepare a Gram stain, one of most important techniques for the identification of bacterial types. You will examine the stained bacteria to identify the common shapes and arrangements of the cells.

You will also gain experience using staining techniques that highlight specific features of bacterial anatomy. Our knowledge of bacterial anatomy has been greatly expanded through use of the electron microscope. Yet, the light microscope remains the principal tool for observing bacteria in clinical and research laboratories, and when coupled with staining techniques, the microscope will reveal many details of bacterial cell structure. Staining techniques are available that allow visualization of many features of bacteria, such as flagella, capsules, cell walls and intracellular granules. In this exercise, you will learn staining techniques for detecting bacterial endospores, and examine demonstration slides showing other features of bacterial anatomy that have been selectively stained.

Summary of exercise

1. You will learn how to prepare a bacterial "smear" on a microscope slide and prepare Gram stains for your stock culture bacteria.
2. Gram-stained bacteria will be examined and the shape and arrangement of the cells will be elucidated.
3. The Shaeffer-Fulton stain will be used to stain for endospores.
4. You will use two techniques for studying bacterial motility.
5. You will observe some other staining techniques on demonstration slides.

I. "Fixing" Bacteria and making a bacterial "smear"

Most staining procedures require bacteria that are evenly spread out and "fixed" to a glass microscope. Fixing means that the slide is heated moderately to kill the cells and cause them to bind to the glass slide. The result is called a bacterial smear.

Supplies

broth cultures of:

Bacillus subtilis

Proteus vulgaris

Enterococcus faecalis

Pseudomonas aeruginosa

cap color

green

pink

purple

brown

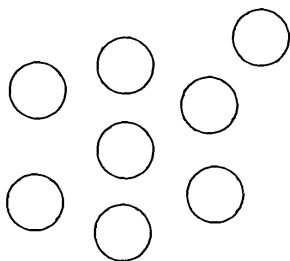
You should Gram stain *B. Subtilis* and *E. coli* first.

Escherichia coli and *Staphylococcus simulans* (on plates)

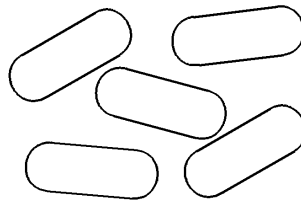
your bacterial unknown

CLEAN microscope slides

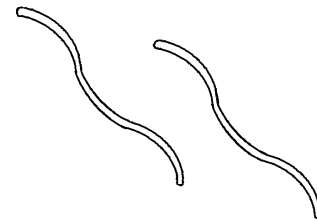
Figure 1A. Common shapes of bacterial cells



COCCUS (pl. COCCI)

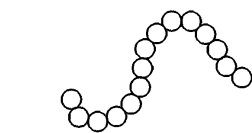


BACILLUS (pl. BACILLI)

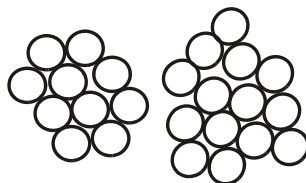


SPIRILLUM (pl. SPIRILLA)

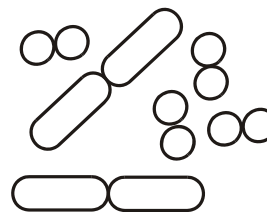
Figure 1B. Common arrangements of groups of bacteria.



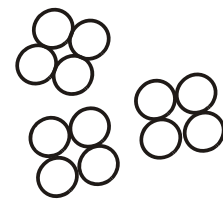
STREPTO- (chain)



STAPHYLO- (group)



DIPLO- (pair)



TETRAD

Notes: 1) Most bacteria are “**randomly**” arranged, i.e., having no specific arrangement.

2) Bacilli never occur in a staphylo arrangement.

Preparing a bacterial smear

1. Clean the microscope slides THOROUGHLY with soap and water before using them.
2. If the smear is to be made from a culture on solid medium, then first place a small drop of water on the slide using your inoculating loop. If the cells are from a broth culture, this step usually can be omitted.
3. Flame sterilize the loop, and let it cool briefly.
4. Aseptically transfer the inoculum from the culture to the slide. If the inoculum is from a solid culture, suspend the cells in the drop of water.
5. Spread the inoculum over a dime-sized area on the slide.
6. Allow to air-dry.
7. Pass the dry slide through a flame to "fix" the bacteria.

Helpful hints for fixing bacteria

1. Do not put too much bacterial growth on the slide. This is frequently a problem when using bacteria grown on solid medium. Ideally, you only need enough cells to cause the water drop to appear SLIGHTLY turbid.
2. Do not speed up the drying process by holding the slide over the Bunsen burner, this frequently leads to bacterial "soup" which will not stain correctly.

I. THE GRAM STAIN

The Gram staining procedure was developed in 1883 by Hans Christian Gram and remains today the most important and widely employed staining technique. The Gram staining procedure often serves as the first step in identifying unknown bacteria by allowing classification of bacteria into two major groups. Some bacteria (called **Gram-positive**) are stained purple as the dye **crystal violet** binds to their cell walls. This dye washes from cell walls of other bacteria (called **Gram-negative**), which are then stained red with a counterstain called **safranin**.

Supplies

Crystal violet

Safranin

Microscope slide containing fixed bacteria

Lugol's iodine solution

Squirt bottle of dH₂O

Ethanol (ETOH)

Rinsing tray

Preparing a Gram stain

Prepare Gram stains for the organisms listed in Table 1. Record your observations in Table 1. Figure 1 shows the common shapes and arrangements of bacteria.

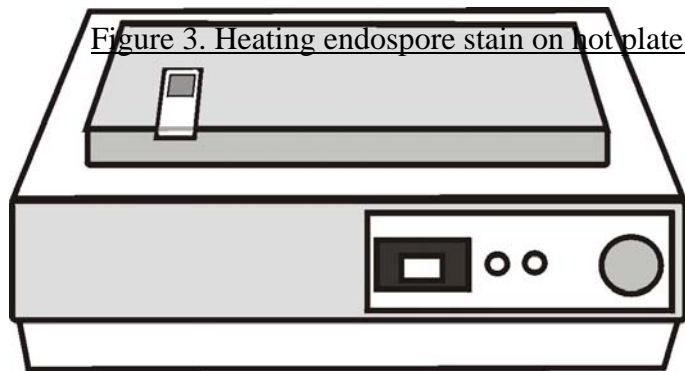
1. Cover the fixed smear with Hucker's Crystal Violet (the PRIMARY STAIN) for ONE MINUTE, then rinse with water.
2. Cover the smear with Lugol's Iodine, a MORDANT, for ONE MINUTE, then rinse with water. This reagent causes binding of the crystal violet in the cell walls of Gram positive bacteria.
3. Decolorize the smear with 95% ethanol (ETOH) until no more dye is removed, then rinse immediately with water.
4. Cover the smear with Safranin solution (the COUNTERSTAIN) for 30 seconds, then rinse with water and allow to air dry (or very carefully blot dry).
5. Examine the slide under the microscope WITHOUT cover slide.
6. Use an ocular scale to measure the length and width of your unknown. Measure 5 cells and calculate an average; and make a large representative drawing of the cells.
7. Record your observations in Table 1.

Important considerations when preparing a Gram-stain

1. It is recommended that the first two cultures you Gram stain are *Bacillus* and *E. Coli*.
2. The most common problem causing an erroneous Gram stain is large cell clumps in the smear, which often do not destain properly. Over-destaining of clumps can cause Gram-positive cells to appear Gram-negative; and “bleeding” of excess stain from clumps and cause Gram-negative cells to appear gram-positive.
3. Destaining is the most critical step of the procedure. Pay particular attention to the demonstration given by the instructor.
4. The Crystal Violet solution should be a homogenous solution. Test your solution initially on a clean slide. If the stain appears as a suspension of particles, it must be replaced with fresh reagent.
5. Make sure that your smear is completely dry before adding immersion oil to the slide.
6. The age of bacterial cultures is also important, [particularly for Gram positive rods. The cell walls of some species, such as *Bacillus*, lose the ability to retain crystal violet as the culture begins to age, and the cells appear Gram-negative instead of Gram-positive. Thus, using fresh cultures is important when preparing a Gram stain. Even so, certain bacteria, such as certain species of *Bacillus* may appear as a mixture of Gram-types even in relatively young cultures.

II. Staining bacterial endospores

Endospores are produced within the cytoplasm of bacterial cells in a few genera of bacteria, such as *Bacillus* and *Clostridium*. When fully mature the endospores allow the cell to withstand adverse conditions, such as desiccation, nutrient deprivation, extremes in temperature, chemical agents including antibiotics, and ionizing radiation. A mature endospore exists in a "cryobiotic" state, meaning that there is no measurable metabolic activity. Their extreme resistance to adverse environmental conditions explains why endospores can remain viable for many decades or longer.



During the initial stages of formation the endospore remains within the original cell, but the cell subsequently degenerates and releases the endospore. Staining of endospores is facilitated by the unique properties of the endospore cell wall. The technique that we will use is called the SCHAEFFER-FULTON endospore stain. This procedure uses heat to drive the dye MALACHITE GREEN into the endospores. After staining, the endospores will retain the dye while normal cells are easily destained with water. The unstained cells are visualized using safranin as a counterstain.

Supplies

Bacillus subtilis, grown on TSA plate
Unknown, week old culture grown on slant
malachite green stain (on side bench)

safranin counter stain
pieces of paper towel

Procedure for staining endospores

1. Prepare a bacterial smear and heat fix in the usual manner.
2. Cut a piece of paper towel slightly smaller than the size of a cover slide.
3. Place the piece of paper towel over the smear and flood it with malachite green. The paper towel should be fully saturated with some free stain on the surface.
4. Place a cover slide over the paper towel. (This will reduce evaporation during the next step.)
5. Place the slide on the heater plate (approx. 90°C) for 5 minutes. If it looks as though the paper towel may completely dry at any point, carefully add more stain so that it runs under the cover slide.
6. Using your slide clamp, remove the slide from the heater plate and allow the slide to cool. Remove and dispose of the cover slide in your disposal jar. Rinse off excess dye with water and discard the paper in the garbage.
7. Counter stain with safranin for 30 seconds.
8. Rinse, allow to dry, and examine the cells under the oil immersion lens. Record your observations in the space provided at the end of this section. Endospores will appear green and vegetative cells will appear red.

III. DETERMINING BACTERIAL MOTILITY

Bacterial flagella are too small to be seen with a light microscope unless they are stained in a way that makes them visible (such as the Liefson's flagella stain demonstrated in Part V). However, motility can be determined directly in several ways, such as by using wet mounts and culturing the cells in Motility agar. Both techniques have advantages and disadvantages.

Supplies

4 tubes of Motility Agar (blue caps)
microscope slides, cover slides, & vaseline
Proteus vulgaris (pink cap)
Pseudomonas aerogenes (brown cap)
broth culture of *Enterococcus faecalis* (purple cap)
your bacterial unknown

A. Determining motility using motility agar

In this method, the bacteria are inoculated into a tube of culture medium by stabbing vertically through the center of the medium (Figure 4). The medium is called a **soft agar deep** because it is not slanted and contains 0.5% agar instead of the standard 1.5%, which allows motile bacteria to more easily move through the medium. This technique does not work well for bacteria that are obligate aerobes, which will only grow on or near the surface of the medium, but it does distinguish motility from Brownian motion (see below), which does not cause enough significant movement of

Figure 4



bacterial cells within the agar medium.

Procedure for using Motility Agar

1. Stab *Proteus*, *Enterococcus*, *Pseudomonas* and your unknown into motility agar "deeps" using your inoculating needle.
2. Incubate the cultures at 37°C for 24 hours. Record results of motility agar cultures.
3. Bacteria testing positive will form a diffuse zone of growth extending outward from the line along with the stab line; a negative result will appear as a distinct line of growth only right along at the stab line. Obligate aerobes will only grow near the surface of the medium

B. Determining motility using a wet mount (use same organisms as for motility agar)

A wet mount can be used to directly observe bacterial motility; however, there are some difficulties associated with using wet mounts. Bacteria in a wet mount do not have much contrast with the surrounding medium– you may at first have difficulty in seeing the cells. Furthermore, bacterial motility must be distinguished from **Brownian motion** (the random vibrational movement of small particles in a liquid medium) and **Convection Currents** (the mass flow of fluid due to temperature differential or surface tension). Mass flow along the edge of a water droplet will cause all bacteria to move in the same direction. Even non-motile bacteria display Brownian motion, but only motile bacteria will display a prolonged, directional movement indicative of true bacterial motility.

Procedure for preparing a wet mount

1. Place a small droplet of the sample to be observed on the microscope slide. If starting with bacteria grown on solid medium, suspend it in a small droplet of water.
2. Anchor the cover slip to the slide with a thin bead of vaseline, as shown in Figure 5 and demonstrated in class.
3. To find the ‘focal plane’ in which the bacteria occur, first focus on the edge of the water droplet, then look off to the side to find the bacteria.
4. Bacterial motility is most reliably assessed around the perimeter of the water droplet or near an air bubble, since this where oxygen is most abundant. After first finding the right focal plane under low power, make observations using the **dark field adapter** with the high dry lens. Then, rotate the low power objective back into place, remove the dark field adapter, and then make observation all the way up to the oil immersion lens.
5. **Slides containing wet mounts should be disposed of in the disposal jars on your bench. Remember, they contain living bacteria.**

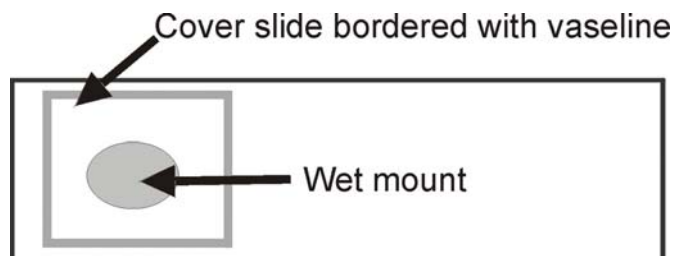


Figure 5. Wet mount for observing bacterial motility

IV. OTHER STAINING TECHNIQUES AND BACTERIAL STRUCTURES

Other staining techniques are used to demonstrate special bacterial structures or cell types. A number of examples are available for you to examine and draw.

1. Flagellation– *Proteus vulgaris* flagellation. Flagella are normally too thin to be seen with a light microscope. Liefson's flagella stain can be used to demonstrate their presence. During the procedure, the stain becomes deposited upon the flagella until they are thick enough to be seen. What type of flagellation do these cells possess?

2. Capsule– *Flavobacterium capsulatum* The capsule is a gelatinous layer outside of the cell wall, often much thicker than the cell itself. This slide illustrates a technique called '**negative staining**'. In this technique, the stain binds to the bacterial cells and to the residual culture medium adhering to the microscope slide, but not to the capsules themselves. The result is that the capsules appear as clear zones surrounding the bacterial cells.

3. Spirillum– *Spirillum volutans*. Spirilla are bacteria with a short helical cell shape. Cells of many species are very small and only can be readily observed by using of dark field microscopy. *Spirillum volutans* has relatively large cells. What type of flagellation do these cells possess?

4. Spirochete– *Treponema pallidum*. This organism, the agent of syphilis, is an example of a spirochete. It is helical like a spirillum, but the twists are much tighter and the cell is much longer. As mentioned in class, spirochetes possess a complex cell structure.

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Name: _____

Indicate where your results do not agree with your expectations, and write a brief explanation of the discrepancy

Table 1. Observations of Gram stained bacteria

ORGANISM	Gram reaction (positive or negative)	Cell shape	Cell arrangement
<i>Escherichia coli</i> (on plate)			
<i>Staphylococcus simulans</i> (on plate)			
<i>Bacillus subtilis</i> (green cap)			
<i>Proteus vulgaris</i> (pink cap)			
<i>Pseudomonas aeruginosa</i> (Brown cap)			
<i>Enterococcus faecalis</i> (purple cap)			
Your semester unknown (From fresh culture)			
Unknown #1 Name:			
Unknown #2 Name:			
Unknown #3 Name:			

Size of unknown: Width measurements _____ Avg: _____ μM

If not a coccus, also measure the length:

Length measurements _____ Avg: _____ μM

Make a large drawing of a several cells of your semester unknown showing accurately showing their shape and arrangement.

Results of Endospore Stain Procedure.

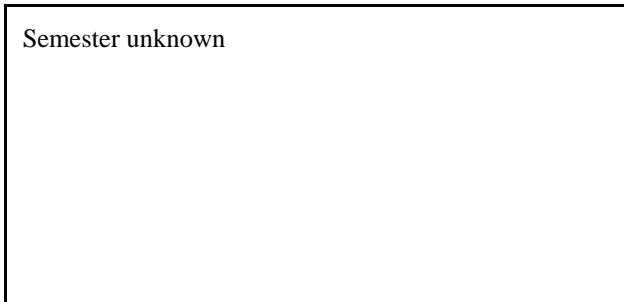
Draw representative examples of *Bacillus* cells and endospores. Label cells with and without endospores (if present), and endospores free of cells. Drawings must be large enough to show sufficient detail, and accurately reflect relative sizes of cells and endospores.



What was the **shape** of the endospores?
round / elongated / oval

What was the **position** of the endospores within the bacterial cells? center / near end / not observed

Draw representative examples of the endospore stain for **your unknown**. Label appropriately. If spores are present, what is the:



What was the shape of the endospore?
round / elongated / oval

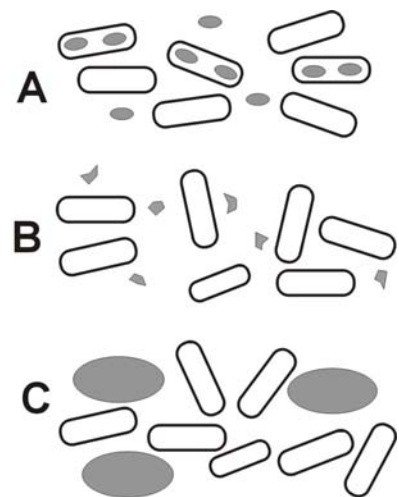
What was the position of the endospore within the cell? center / near end

Turn in typed responses to these questions.

1. Explain if each of these conditions would alter the results of the Gram stain for a Gram-positive bacillus, and identify what the final gram stain result would be when:

- 1) over-destaining with ethanol
- 2) using a two week-old culture
- 3) using a heavy, clumpy smear of bacteria
- 4) forgetting the safranin step.

2. The diagram to the right shows drawings of Schaeffer-Fulton endospore stains prepared by three students. In each case, the shaded structures were identified as endospores. But you know better! Explain why the stained structures in each of these samples could not be endospores.



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Draw and briefly describe the appearance of the cultures in the motility agar. Label the position of the bacterial growth in each tube.

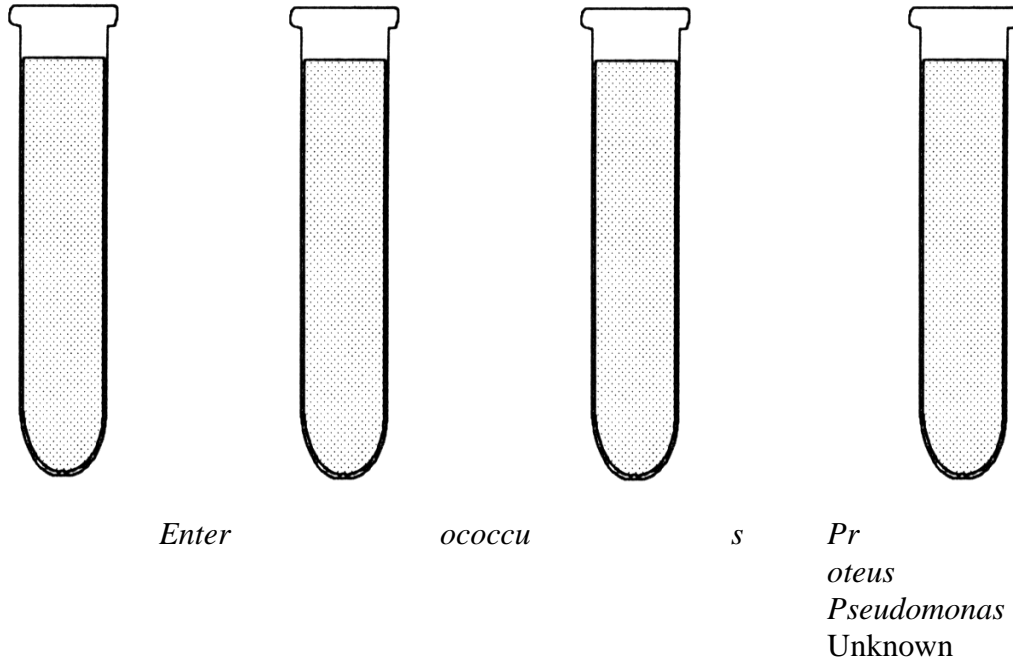


Table 2. Results of motility test

Species	Were results '+' or '-' or NI (not interpretable) for	
	Motility agar	Wet mount
<i>Enterococcus faecalis</i>		
<i>Proteus vulgaris</i>		
<i>Pseudomonas auruginosa</i>		
your unknown		

Indicate if each organism is motile or nonmotile? **Explain any discrepancies**, or if results were NI, explain why.

Enterococcus faecalis: _____

Proteus vulgaris: _____

Pseudomonas auruginosa: _____

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Unknown: _____

DRAWINGS OF PERMANENTLY MOUNTED BACTERIA

This exercise is intended to test your ability to draw exactly what you see, and not a schematic representation. For each sample, make LARGE drawings of **representative cells that convey accurate cellular detail**. DO NOT draw the cells within a circle – this is not necessary.

1. Flagella Stain (*Proteus vulgaris*). Find a well flagellated cell and accurately **draw the flagella as they appear, not schematically**.

What is the arrangement of the flagella on these cells? _____

2. Spirillum cell form (*Spirillum volutans*)

What is the arrangement of the flagella on these cells? _____

3. Spirochete cell form (*Treponema pallidum*) Draw large enough to show detail.

4. Capsule stain (*Flavobacterium capsulatum*)

Make a drawing a region of the slide with a few cells.

***** Be sure to also turn in the completed Semester Unknown Summary – I *****