SSt Simple Staining

Learning Objectives

The student will

- Use aseptic techniques.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments when making observations.
- 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

A brightfield microscope creates an image by directing light from the illuminator at the specimen; this light is differentially transmitted, absorbed, reflected, or refracted by different structures. (OpenStax CNX, 2018) This alteration of light as it passes through the specimen and into the lens system of the microscope creates the image. Bacterial cells are so small and thin that they alter the light very little and, consequentially, are virtually transparent or invisible. In other words, the cell lacks contrast with the background. Staining the specimen will artificially create contrast so that cells may be visible. Along with magnification and resolution, contrast is the third element that contributes to the amount of detail that can be observed.

Some staining techniques involve the application of only one stain to the sample; others require more than one. In simple staining, a single stain or dye is used to emphasize particular structures in the specimen. A simple stain will generally make all of the organisms in a sample appear to be the same color, even if the sample contains more than one type of organism. (OpenStax CNX, 2018)

Stains, or dyes, aqueous slat solutions. They contain a positive ion and a negative ion one of which imparts the color. The chromogen (sometimes called the chromophore) is the colored ion. If the chromogen is positively charged, the counter ion is the negatively charged hydroxide ion \( \text{OH}^- \) making this type of stain a basic stain. (Here the term basic does not mean elementary. Instead, basic refers to chemical property of having excess hydroxide ions.) Because most cell structures have a negative charge, the positively charged, colored

![Figure 1 A basic stain has a positive chromogen and the negative hydroxide ion. The colorless cell has a net negative charge.](image-url)
chromogen will be attracted to negatively charged cell. The result of this interaction is the cell retaining the color and the background remaining uncolored. OpenStax Microbiology calls this a positive stain, a dye that will be absorbed by the cells or organisms being observed, adding color to objects of interest to make them stand out against the background. (OpenStax CNX, 2018)

If the chromogen is negatively charged, the counter ion is H\(^+\). This makes the stain an acidic stain. In this case the negative chromogen will be repelled by the negatively charged cell and the cell will remain colorless against a dark background. The exercise, Negative Staining, discusses this type of staining process in greater detail.

Because cells typically have negatively charged cell walls, the positive chromophores in basic dyes tend to stick to the cell walls, making them positive stains. Thus, commonly used basic dyes such as basic fuchsin, crystal violet, malachite green, methylene blue, and safranin typically serve as positive stains. (OpenStax CNX, 2018)

Before cells are stained with a basic dye, they must be fixed to the slide so that they do not wash away with the excess stain. The “fixing” of a sample refers to the process of attaching cells to a slide. Fixation is often achieved either by heating (heat fixing) or chemically treating the specimen. In addition to attaching the specimen to the slide, fixation also kills microorganisms in the specimen, stopping their movement and metabolism while preserving the integrity of their cellular components for observation. (OpenStax CNX, 2018)

To heat-fix a sample, a thin layer of the specimen is spread on the slide (called a smear or emulsion), and the slide is then briefly heated over a heat source. (OpenStax CNX, 2018) In this lab, you will use the following procedure to heat fix a sample for staining.

### Smear and Heat Fixing Procedure
1. With a wax pencil, divide a clean microscope slide into sections, one for each sample. Label each section with an abbreviation for each sample. No more than three samples per slide.
2. To make a smear from a broth culture, aseptically obtain a loopful of the culture from the tube and spread it on the slide over an area about the size of a nickel or larger. The larger the area, the more space you will have to view your cells.
3. Making a smear from a sample growing on solid media, can be tricky. First, you will need to add some liquid to the slide first so that you have something to spread the cells in. Placing an entire drop of water on the slide, while not technically incorrect, will delay the process considerably. A full drop of liquid from your water bottle, approximately 50 µl, will take a long time to air dry. A loopful, closer to 10 µl, of water is plenty and will dry fairly quickly. Second, students tend to get way too many cells. So many cells that they remain in large clumps. This creates some problems. Stains cannot make contact with all cells equally leading to erroneous results when using a differential staining technique. In addition, one cannot adequately observe morphology and arrangement if the cells form large clumps and individual cells cannot be seen. The following steps are designed to minimize these difficulties.
a. Squirt some water from your water bottle on the bench top. Place the loop in it to obtain a film of water spreading across the loop. Touch this to your slide, releasing the tiny amount of liquid onto the slide. Because neither the water nor the slide is sterile, this part is not aseptic.

b. Wipe up the excess water on the bench top so that it is not confused with a spill.

c. Now, aseptically, obtain a small amount of cells on your loop by touching the growth on the surface of the agar. (You will need to sterilize your loop first!) Do NOT swipe a bunch of cells. You do not need to see cells on the loop.

d. Touch the loop to the liquid on your slide; do not spread it. You should see some growth come off into the water as cloudiness. Incinerate your loop to remove excess cells from it.

e. After your loop cools, go back and spread the water containing cells into a nickel or larger area.

4. Allow the smear to air dry. Using a flame or other heat source essentially boils the cells distorting size and shape. You will use heat in the next step, but as long as the cells have dried gently first, they will be close to their original dimensions.

5. After the smear has air dried, heat fix it. With a clothes pin, hold the slide near the mouth of the incinerator for 10 seconds. (Alternatively, pass it though the flame two times.)

6. Set the slide on the bench top to cool before staining. I recommend that you stain one slide at a time. Leave the heat fixed slide on the bench and not on the staining rack while working with the next slide.

Simple Staining Procedure

1. Place a rack across a staining tray. The tray will catch excesses stain and rinse water.

2. Place one heat fixed slide on the staining rack. If your extra slides are on the rack, there is the risk of one falling into the staining tray that contains excess stain ruining the smear. I have seen this happen many times!

3. Flood the entire slide with stain and allow it to sit for the required amount of time. In simple staining, think of these times as approximate minimums. It is important to allow the stain to stay in contact with cells for the minimum amount of time so that cells have sufficient time to absorb the stain. Leaving the stain on for a few additional seconds will not affect the result. On the other hand, leaving the stain on for 5 minutes when you only need 1 minute will risk stain accumulating on and between cells obscuring cell morphology and arrangement.

4. Gently rinse off excess stain with an indirect stream from your distilled water bottle.

5. Gently blot with bibulous paper. Blotting too vigorously will unnecessarily remove cells from the slide.

6. Set your slide on the bench top (not on the staining rack) until you are ready to view it under the microscope. You may need to relabel the slide if the wax marks have been washed off.

Cellular Morphology and Arrangement

Simple staining allows one to observe the morphology and arrangement of the bacterial cells. Morphology refers to “form” or shape. We can use terms like spiral, bacilli (rod shaped), and cocci (spherical) to describe cell shapes. Keep in mind that the bacterial cells came first and these categories are our attempt to describe them. A given cell may not fall neatly into one category. For example, some cells are short and “fat,” neither cocci nor rod shaped. This cell may be referred to as coccobacilli.

By arrangement we refer to cells’ relationships to other cells. Cells of a given species tend to arrange themselves in a typical pattern. Cells may form chains (strepto-), small groups of two (diplo-), four (teta-), eight (sarcina), large grape-like clusters (staphlo-) or parallel bacilli (palisades). Some bacteria exist as single cells, visually unattached to other cells. Cell arrangements depend on
how the cell divides and how the culture is grown. After division, daughter cells may completely separate or remain attached. Cells also divide in a characteristic plane. If successive divisions occur parallel to one another and daughter cells remain attached, the result is a chain. If they divide perpendicular to each other, the result may be a tetrad or sarcina form. If the plane of division is random, grape-like clusters may form. A species grown in a broth may appear to have a different arrangement compared to the same species grown on solid media.

How well you spread out the cells on the slide also influences the microscopic appearance. You will not be able to correctly determine cell arrangement if the smear is too thick and all you see is an amorphous lump. This is another reason you want a thin, well spread out smear.

When recording observations of morphology and arrangement of a pure culture, you will want to form an opinion on the most prevalent arrangement. It is also permissible to record more than one arrangement if that is what you observe. The important thing here is that you record what you actually observe under the microscope. Unless your smear is too thick, you will not be penalized for cells that do not appear arranged as we expect. If, however, your drawings and/or observations do not match what is seen through the microscope lens, your scientific credibility may be questioned.

**Cell Drawings**

Making accurate observations is one of the learning objectives of this course. Accurate drawings are an important part of your observations. Follow these guidelines for drawing cells.

- Because details of the microscopic appearance are important, your drawings will **not** be to scale. When you make a drawing, turn your attention to the group of cells you are drawing and magnify it in your mind’s eye. Cells should be drawn large enough so that you can show an accurate shape and arrangement. Each cell should measure about 4 mm in diameter on your paper.

- Your drawing should capture the **relative length and width** of the cells. Most cocci are not perfect spheres.
Some are a bit elongated. If this is what you see, try to capture that. Some bacilli are long and skinny and some are short and stubby looking.

- There should be no pointy corners, no open circles, no extra “tails” and no overlapping rings.

Take a little extra time to think about these things when drawing. If you think you are looking at bacillus with a pointy corner or a flagellum, check with the instructor. If this is something real, the instructor does not want to take off if you are making accurate observations. Your instructor also wants to make sure you are looking at cells and not dust or other debris on the slide.

**Experiment/Exercise**

**Materials per student pair**
- 4 microscope slides
- Safranin stain
- Methylene blue stain
- Crystal violet stain

**Cultures**
- Fresh overnight broth cultures
  - *E. coli*
  - *Staphylococcus epidermidis*
- Cultures on solid media (environmental zig zag plate and/or other pure culture t-streak plates)

**Procedure**
1. Each person will make 2 identical slides as follows.
2. Either you or your partner will divide a slide in half with a wax pencil. On one side place a smear from the *E. coli* broth. On the other side place a smear using one of the pure culture t-streak plates or the environmental sample (SI exercise). REMEMBER the procedure is different for taking cells from solid media versus liquid media. Let both smears dry BEFORE heat fixing. See the section Smear and Heat Fixing Procedure. Repeat for the second slide.
3. The other person will make 2 slides, each with *S. epidermidis* broth on one side and a different pure culture from a plate on the other side.
4. Each person pick a different stain and stain ONE of their slides according to the following times:
   - safranin stain, 1 minute
   - methylene blue stain, 2 minutes
   - crystal violet stain, 1 minute
5. Find cells form each smear under oil immersion using your assigned microscope. Be sure to start with the scanning objective and follow the procedure given in Microscope Theory. (Your partner will view their slide with their microscope.)
6. When you find cells on oil immersion the first time, call your instructor (or GTA or ULA) over to verify that you are looking at cells. They will initial your Data and Observations sheet.

7. Draw a representative group of cells and record the color, morphology and arrangements of the groupings that you drew. Use the technical terms given above, if applicable. If cells are not attached to other cells, write “singles” for the arrangement. Your drawings must be neat!

8. Allow your partner to look through your microscope and make the observations of your cells while you make observations of their cells. Do not copy from your partner’s observations sheet.

9. Find cells in the other pure culture smear under oil immersion. You do not need initials this time. You may ask an instructor for verification if you are unsure that you are looking at actual cells.

10. Make the same types of observations of your second smear. Then trade positions with your partner so that you can observe their cells.

11. When completed, you should have drawings and observations of 4 total organisms.

12. Follow the disposal procedure for stains in LS under disposal item 8.
Lab Report: Simple Staining

Name ______________________________
Lab Section __________

Data and Observations

<table>
<thead>
<tr>
<th>Organism (Be sure to write the name correctly.)</th>
<th>Stain used and color</th>
<th>Drawing (See section on Cell Drawings for guidelines.)</th>
<th>Morphology and Arrangement</th>
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Post Lab Questions

1. Critique your cell drawings. Are they large enough?
   Do they capture the relative length and width of the actual cell?
   Did you draw pointy corners?
   Outward tails?
   Inward tails?
   Open circles?

2. The stains used in this exercise are basic stains. What is the counter ion? What charge does the counter ion have? What charge does the chromogen have?
3. What microbial characteristics can one ascertain from a simple stain?

4. A student is directed to make a simple stain of *E. coli* with crystal violet, but got mixed up and used safranin instead. How would their observations be different? Would the information you get be any different?

5. If you dried your smear by placing it near the incinerator instead of air drying it, how might your observations be different?

6. How is the procedure different when taking cells from a solid medium compared to taking cells from a liquid medium? Why is it important that your smear be thin?

7. What is the difference between colony morphology and cellular morphology?
References
