MCB 3020L **Lab Experiment 2**

The Gram Stain

A one lab session experiment

In most microbiological staining procedures, the bacteria are first fixed to the slide by the **heat fixed smear**. In this procedure living, potentially pathogenic bacteria are smeared on the glass slide and allowed to dry. Then the slide is heated so that the bacteria are killed and stuck (coagulation of surface proteins) to the slide. This renders the slide safe and keeps the bacteria stuck to the slide so that simple or complex staining and washing procedures can be carried out without washing them off of the slide.

After preparation of the heat fixed smear of a bacterium, the cells may then be stained. Stains are dyes which stick to cells or cell parts by virtue of their charge or solubility properties. Most simple stains are dyes that have a strong positive charge; that is they are cations. Most proteins, carbohydrates, nucleic acids have a net negative charge. By adding the stain to the cells, ionic attraction keeps the dye molecules attached to the cells so that rinsing with water does not rinse off the stain attached to the cells, but does rinse the stain off the glass (background).

In 1883 Hans Christian Gram invented an important differential staining method that is extensively used today. The stain is called the **Gram Stain**. This staining procedure differentiates microbes into two basic groups: Gram positive microbes and Gram negative microbes. Differential stains render one type of microbe one color and other types of microbes another color. In the Gram stain, Gram positive organisms retain the primary dye complex (crystal violet-iodine) and appear blue/purple whereas Gram negative cells loose the primary dye complex during the challenge rinse (acetone or alcohol) and are stained by the counter-stain, safranin which makes them pink.

Most differential stains have a challenge step that follows staining with a primary dye. In the Gram stain the challenge step is a rinse with either ethanol or acetone (either may be used). This step dehydrates and tightens the carbohydrate structures such as the cell wall peptidoglycan. For Gram positives the rinse does not enter the cell. But, Gram negative cells have mainly a lipid cell wall (even though they do contain a very thin layer of peptidoglycan) that allows the challenge rinse, acetone, to penetrate the cell and rinse out the crystal violet-iodine complex rendering the Gram negative cell colorless. Thus, the Gram negative cells must be stained another color to be seen, and this is done with the counter-stain.

The counterstain used in the Gram stain is Safranin (pink). This is applied after the challenge step and stains the Gram negatives pink. To a Gram positive cell the counterstain stains the cell, but the cells are already so heavily stained by crystal violet-iodine complex that the addition of pink does not change the dark blue color of the cells. Thus Gram positives appear deep blue/purple and Gram negatives appear pink.

Note that the success of the Gram stain relies upon the integrity of the cell wall. Gram positive bacteria that have been overly heat fixed resulting in destruction of all or parts of their cell wall can appear to be pink or have pink areas, but this is an artifact! Further, old or dead cells of Gram positive bacteria can appear pink. This is because the cell wall has allowed the challenge rinse to enter the cell. Successful Gram stains should be done on young, growing cells. Many experiments in this course will utilize the Gram stain. You should KNOW THE GRAM STAIN by heart!

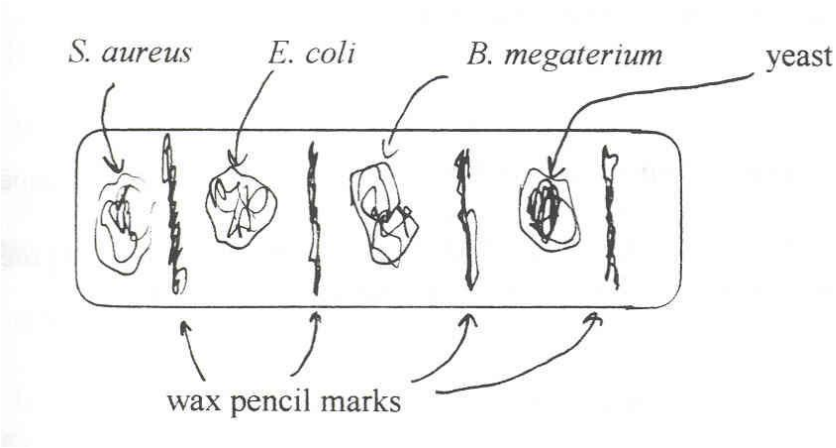
Materials per pair

1. Slant cultures of *Escherichia coli, Bacillus megaterium, Staphylococcus aureus* or other bacteria.
2. Inoculating loops, slides.
3. Yeast suspension.
4. Gram staining reagents in dropping bottles, and staining trays.
5. Student samples.

Procedure

Heat Fixed Smear

1. Obtain three **dry clean glass slides**. If the slides are not clean (hold it up to the light and look) clean them with detergent and water, then dry them. Be sure your slide is not multiple slides stuck together. In this experiment you will need to prepare **three** heat fixed slides; two will contain four organisms on each slide. The third will be a heat fixed smear of tooth scraping. The following directions are for one slide.
2. Heat the slide gently by passing it through a Bunsen burner flame. Mark off four sections with a wax marking pencil as shown below:



3. Place three drops on the slide from ONLY one loopful of water. The instructor will demonstrate this.
4. Flame sterilize a loop.
5. With your other hand pick up the slant, open the top holding the cap with the little finger of the hand holding the loop. Flame off the top of the tube.
6. With the sterile loop, touch the bacterial growth on the slant. Flame off the tube and replace the cap.
7. Smear the bacteria on the end of the loop into one of the drops of water on the slide.
8. Repeat steps 4-7 for each slant culture.
9. For broth cultures (yeast suspension), after flame sterilizing your loop, simply place a loopful of culture directly onto the slide (not into a drop of water).
10. Allow the smears to air dry.
11. Pass the dry slide slowly through the flame three times. The object is to heat the slide to about 70oC. This can not be accurately measured, but can be tested. The slide should be very hot to the touch, but touchable.
12. For the tooth scraping, place a part of a loopful of water on a slide. With a toothpick, scrape around and between your teeth and smear the material into the drop of water. Dispose of the

toothpick in a Biohazard container. Allow the smear to air dry, then heat fix as above.

13. For your sample, you can smear it directly on to the slide or you can make a suspension. You need to figure out how to get your material onto the slide as a smear so that you can do the heat-fix.

Gram Stain

1. Stain the smears with Crystal Violet for 1 minute.
2. Rinse with a mild stream of water.
3. Stain the smears with Gram's Iodine for 1 minute.
4. Rinse carefully with acetone. **CAREFUL !!!** Rinse only until blue color stops coming out of the smear. DON'T **over-rinse!**
5. Rinse off the acetone with water.
6. Counter-stain with Safranin for at least 10 seconds.
7. Rinse with water, air dry. Remember, **the slide must be completely dry before putting it on your microscope**.
8. Use the low power (10X) lens to find a good field (this is an area with single cells separated from each other so that you can see individual cells). Add a drop of oil and immerse the 100X oil immersion lens into the oil by rotating the nosepiece. Use only the fine adjustment to bring the image into clear focus.

Questions

1. If your organisms were all blue with pink spots, what is the Gram reaction? Why?
   If your organisms were all pink but had some blue spots, what is the Gram reaction? Why?

2. How can your answer to question 1 be proven? (Think of something that can be done in the lab.)

3. What would be the reason(s) for not finding any organisms on the slide? What could have gone wrong in the procedure?

4. What would you expect from a Gram stain done on a slide that was heated too HOT during the heat-fixed smear?

Textbook Ref: Brock: Biology of Microorganisms, Section 4.1

PREPARATION for NEXT LAB (Titers).

The next lab will titer (pronounced tight-er) something YOU WILL BRING IN. It should be something that has a lot of microbes in it. Spoiled food would work, soil, dog or cat feces, vacuum cleaner stuff, slime off of shower curtains, etc.....be creative. Experiment 3 will introduce you to the techniques of serial dilution and plating out your sample so that you will be able to count the number of viable bacteria in your sample. It is important to know what is going to happen well in advance. Students bringing in a sample will earn TWO bonus points.