LAB 2
Bacterial Staining Techniques I

I. Complete Lab 1
II. Staining Microorganisms: Simple Stains (Direct and Negative)
III. Morphological Unknown

I. Complete Lab 1:

Collect your plates from the trays on the side bench. Observe the TSA plates for colonies of various sizes, shapes and colors. Each bacterial or fungal species gives a characteristic colony color and morphology. Draw the colonies observed on both TSA plates in the spaces provided in the Results section of Lab #1. Pick three colonies from either of the TSA plates and describe the colony color and morphology. Also observe the cloudiness (turbidity) of your nutrient broth tube and estimate the number of bacteria per mL (see turbidity table below).

TERMS AND DEFINITIONS

Colony: a single cell divides exponentially forming a small, visible collection of cells. Colonies are observed when bacteria are grown on a solid medium. Each colony usually contains 10^7-10^8 bacteria.

Colony morphology: Characteristics of a colony such as shape, edge, elevation, color and texture.

Turbidity: cloudy appearance of a liquid medium due to the presence of bacteria. You can "estimate" the number of bacteria per mL by using the table below.

Turbidity	# Bacteria per mL
none	0 - 10^6
light	10^7
moderate	10^8
*heavy	10^9

*Usually bacterial populations do not exceed 3 x 10^9 bacteria/mL when grown in liquid media.

II. STAINING MICROORGANISMS

A. Smear preparation
Simple Stains:
B. Direct stain
C. Negative stain

TERMS AND DEFINITIONS

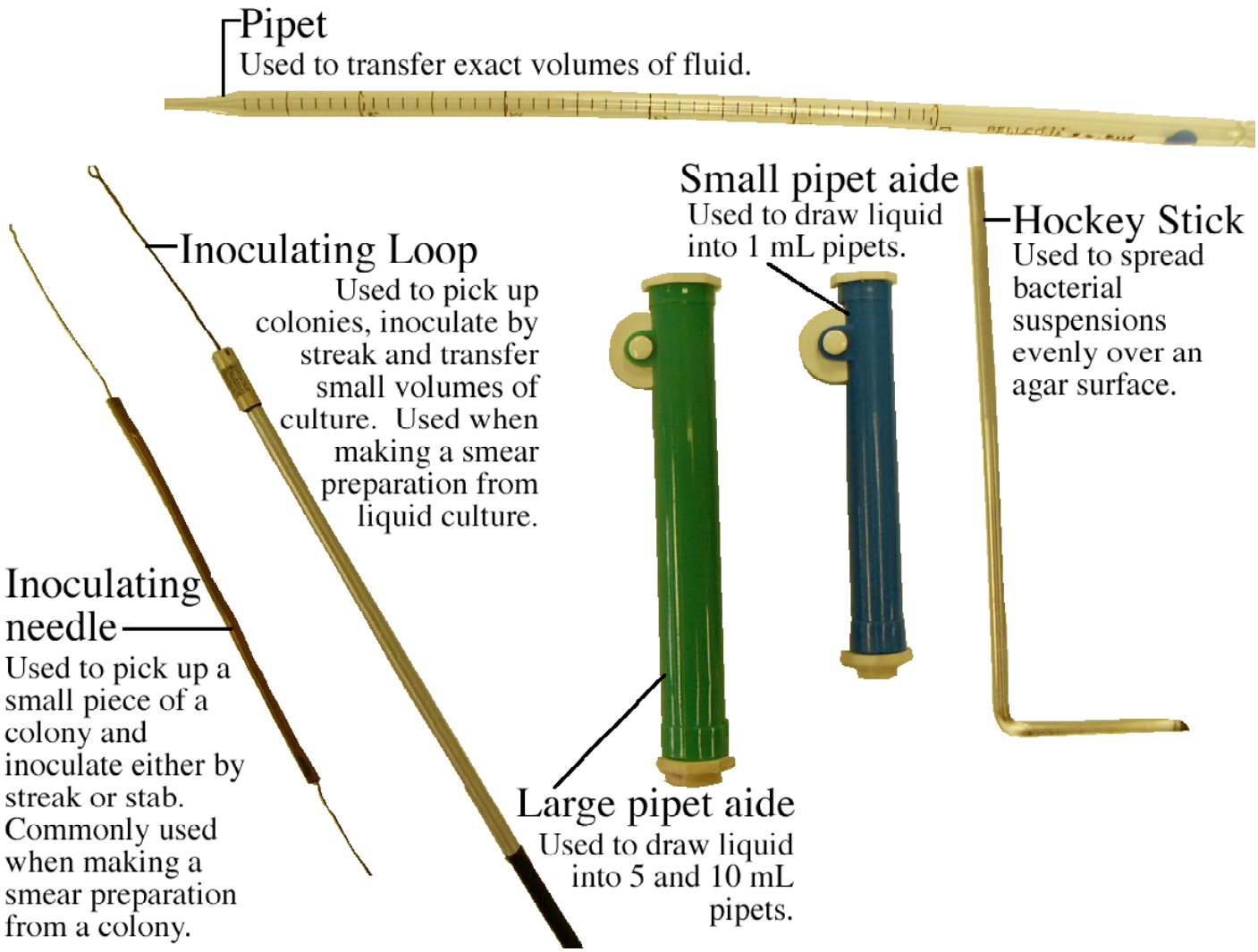
Chromophores: Groups with conjugated double bonds that give the dye its color.

Direct, **cationic**, **basic** or **positive** dyes: contain positively charged groups. Examples include methylene blue, basic fuchsin, and crystal violet. These dyes directly bind to and stain the negatively charged surface of bacterial cells.

Negative, anionic, or acidic dyes: contain functional groups that have a negative charge. Examples include eosin, nigrosin and Congo red. These dyes are repelled by the negatively charged surface of bacterial cells. Thus, they stain the background, leaving the bacterial cells clear and bright against a dark background.

Heat Fixation: application of heat to a bacterial smear preparation. This procedure simultaneously kills and attaches the bacteria to the slide.

MICROBIOLOGIST'S TOOLS



A. Smear Preparation

The first step in most bacterial staining procedures is the preparation of a smear. In a smear preparation, cells from a culture are spread in a thin film over a small area of a microscope slide, dried, and then fixed to the slide by heating or other chemical fixatives. A good smear preparation should be...

1. A **thin layer** of cells so that individual cells can be observed.
2. Fixed appropriately to allow repeated washings during staining.

PROCEDURE: (EACH STUDENT)

Note: A good smear preparation is the key to a high-quality stain. Care taken when creating a smear will allow for accurate observations.

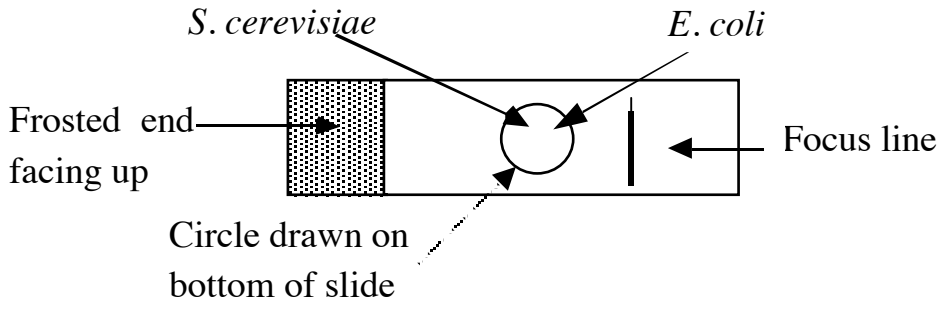
1. Use a slide from your slide box. If necessary, clean the slide using soap and water. Dry the slide using a KimWipe. Place the **frosted side of the slide facing up** and draw a circle (about the size of a nickel) on the **bottom (unfrosted)** side of the slide. Place 2-3 loopfuls of water on the slide. Don't forget to draw a focus line on the top of the slide.

2. Flame an inoculating needle and allow it to cool. Pick up a "tiny" amount of an *Escherichia coli* colony and mix it into the drop of water on the slide.

3. Flame the needle and transfer a small amount of a *Saccharomyces cerevisiae* colony in the same manner to the **SAME** drop.

   You will now have a **mixture** of *E. coli* (bacteria, procaryotic cell) and *S. cerevisiae* (yeast, eucaryotic cell) in the same smear preparation.

4. Air-dry the slide completely. Heat fix the slide by passing it over the flame 3 times. The slide should be uncomfortable to the skin but not painful. The slide is now ready to be stained as described below.



B. Direct stain

The cell wall of most bacteria has an overall net negative charge and thus can be stained directly with a single basic (positively charged) stain or dye. This type of stain allows us to observe the shape, size and arrangement of bacteria.

PROCEDURE: (EACH STUDENT)

1. Use the smear prepared in the previous procedure. Staining is done at the sink.
2. Add several drops of Methylene blue, enough to cover the smear, and wait 1 min.
3. Rinse the slide with water from the squirt bottle and blot the slide with bibulous paper.
4. Redraw the focus line on the top of the slide if necessary.
5. Focus on the line with the 10X objective – **refer to the microscope focusing procedure described in lab 1.** Once you have focused on the specimen using the 10X objective, move the 40X objective lens into position. Use the fine adjustment knob to bring the specimen into focus. Now use the following procedure to view the specimen using the 100X (oil-immersion lens):
   a. Rotate the nosepiece to the empty slot between the 40X and 100X objectives.
   b. Add a drop of oil to slide where the light passes through. The oil has the same refractive index as the glass slide and thus prevents light loss.
   c. Move the 100X objective lens into position. The lens will be immersed in oil.
   d. The specimen will be out of focus but you will probably see a blurry image. Focus using the Fine Focus knob. Turn slowly 1/2 a turn toward you. If the specimen does not come into focus turn back a 1/2 turn to the approximate starting position and then turn a 1/2 turn away from you. If specimen is still not in focus call your instructor over to help you. Never turn the Fine Focus knob more than 1/2 a turn in either direction. NEVER use the Coarse Focus knob when using the 100X objective!
   e. Once the specimen is in focus, find a field that has isolated organisms. Then while viewing the organisms fine-tune the image by gently adjusting the condenser diaphragm to give the best light and adjusting the fine focus to give the sharpest image. If you have difficulty in bringing the image into view, move the stage adjustment back and forth while focusing.

f. After examining the slide - move the oil immersion objective away from the slide. Clean the objective thoroughly with lens paper (NOT KimWipes!) and lens cleaning solution.

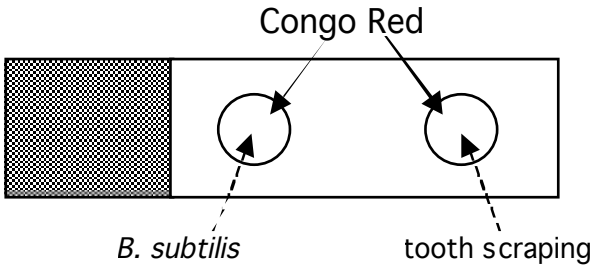
6. Draw the organisms observed in the microscopic field - record in Results Lab 2.
Note: *Saccharomyces cerevisiae* is a species of yeast. It is a relatively large single-celled eucaryotic organism. *Escherichia coli* is a "tiny" rod shaped bacteria (procaryotic).

C. Negative Stain

In contrast to direct stains that bind to bacteria directly, a negative stain colors the background of a smear rather than the bacteria. These stains have negatively charged functional groups so they will not bind directly to negatively charged bacteria. The advantages of negative staining are: 1) bacteria are not heat fixed so they don't shrink, and 2) some bacterial species resist basic stains (*Mycobacterium*) and one way they can be visualized is with the negative stain. However, negative staining does not <u>differentiate</u> bacteria; one can only determine morphology.

PROCEDURE: (EACH STUDENT)

1. Using a flamed inoculating loop, place 2-3 loopfuls of Congo Red in two separate circles on a clean slide. <u>There is no need to add water to the Congo Red</u>.



2. Using a flamed inoculating NEEDLE, pick up a small amount of *Bacillus subtilis* and stir it into one drop of Congo Red.

3. Use a toothpick to scrape material from your teeth near the gumline and stir this into the second drop of Congo Red. Be sure to keep the two drops separate.

4. Air dry - **<u>DO NOT HEAT FIX</u>**.

5. Flood the slide with <u>acid</u>-alcohol (95% ethanol, 3% HCl) until it turns blue. This generally takes ~ 2 seconds. Drain the excess acid-alcohol into the appropriately labeled waste container but <u>do not wash</u> the slide.

6. Allow the slide to air dry; do not blot.

7. Examine both smears. First focus using the 10X objective. You will not be able to see individual organisms, but you should be able to focus on the stain. Then move to 40X and finally to the oil immersion lens with oil.

Note: Organisms appear white (colorless) against a blue stained background. Draw a typical microscopic field for each slide in the Results section of this lab.

III. Morphological Unknown

The staining procedures introduced in Labs 2-4 are commonly used by microbiologists to help characterize and identify bacteria. These stains often make it possible to determine the group of organisms to which an unknown isolate belongs. With few exceptions, staining is the first step in identifying a bacterial unknown. Although staining alone does not give sufficient information about the organism to make a definitive identification, it will give some important clues. You will be given an unknown pure culture on which you will perform the various stains as you go through labs 2-4.

PROCEDURE: (EACH STUDENT)

1. Collect an unknown from the side bench. Record the number of your unknown in the Results section. Your T.A. will also record the number of your unknown. It is important that the same unknown number is used throughout the identification process.

2. Perform a direct stain (methylene blue) on your unknown. Determine the shape of your unknown and any distinctive arrangements of the cells. **Record your observations in the results section following Lab 4.**

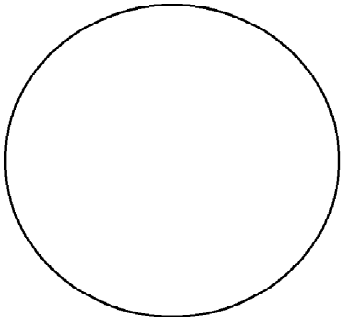
NOTES

LAB 2 RESULTS

II. SIMPLE STAINS

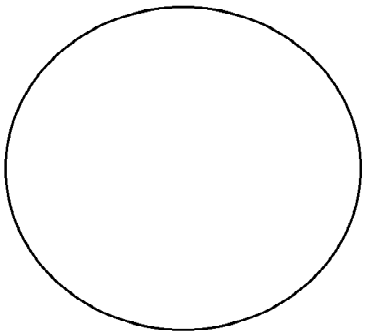
Direct Stain: Draw and label examples of *Escherichia coli* and *S. cerevisiae*. Be sure to illustrate the relative sizes of each microorganism.

E. coli and *S. cerevisiae*

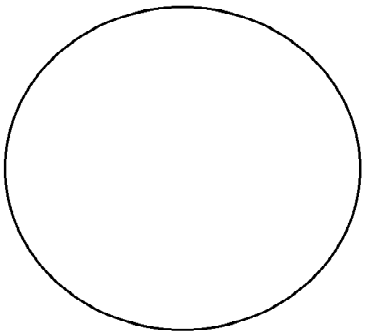


Negative Stain

B. subtilis



tooth scraping



QUESTIONS:

1. What is the purpose of simple staining? _____

_____

_____

2. Differentiate between basic and acidic dyes._____

_____

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_____

3. What is the purpose of heat fixation? _____

_____

_____

4. Is heat fixation done for all stains? Explain. _____

_____

_____

5. What is the major difference between *Saccharomyces cerevisiae* and *Escherichia coli*?

_____

_____

6. When is negative staining used? _____

_____

_____

HELPFUL HINT

You may find it useful to make index cards for each organism, staining procedure, and media encountered throughout this course. At this point, you can record the Gram reaction, morphology, and arrangement for each organism. Later in the course, it will be possible to add further descriptions. The course web site has several features that will assist you in this task.

Although it is common practice to abbreviate the genus by using the first letter (e.g. *E. coli for Escherichia coli*), for the purpose of this class you will need to **write out the entire genus and species name** on practical exams and quizzes. The first letter of the genus is always capitalized, but the species is not. Also, the genus and species name is always typed in italic or underlined.

Organisms introduced in this lab:
Escherichia coli: G- bacilli (rod)
Saccharomyces cerevisiae: (yeast)
Bacillus subtilis: G+ bacilli