LAB 5: DIRECT STAIN AND INDIRECT STAIN

A. INTRODUCTION TO STAINING

DISCUSSION

In our laboratory, bacterial morphology (form and structure) may be examined in two ways:

- by observing living unstained organisms (wet mount), or
- by observing killed stained organisms.

Since bacteria are almost colorless and therefore show little contrast with the broth in which they are suspended, they are difficult to observe when unstained. Staining microorganisms enables one to:

- see greater contrast between the organism and the background,
- differentiate various morphological types (by shape, arrangement, gram reaction, etc.),
- observe certain structures (flagella, capsules, endospores, etc.).

Before staining bacteria, you must first understand how to **"fix" the organisms to the glass slide**. If the preparation is not fixed, the organisms will be washed off the slide during staining. A simple method is that of air drying and heat fixing. The organisms are heat fixed by passing an air-dried smear of the organisms through the flame of a gas burner or holding it in front of the opening of a microincinerator. The heat coagulates the organisms' proteins causing the bacteria to stick to the slide.

The **procedure for heat fixation** is as follows:

1. If the culture is taken from an **agar medium**:

   a. Using the dropper bottle of **deionized water found in your staining rack**, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide (see Fig. 2). Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

   b. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it 2 - 3 times to the drop of water** until the water **becomes visibly cloudy** (see Fig. 3).

   c. **Incinerate the remaining bacteria on the inoculating loop.** If too much culture is added to the water, you will not see stained individual bacteria.

   d. After the inoculating loop cools, **spread the suspension over approximately half of the slide** to form a thin film (see Fig. 4).

   e. Allow this thin suspension to **completely air dry** (see Fig. 5). **The smear must be completely dry before the slide is heat fixed!**

   f. To heat-fix the bacteria to the slide, **pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear near the opening of the microincinerator for 10 seconds** (see Fig. 6) as demonstrated by your instructor. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

2. If the organism is taken from a **broth culture:**

   a. Using a sharpie, **draw a circle about the size if a nickel** on the **bottom** of your microscope slide (see Fig. 10)

   b. Turn the slide over. **Using your sterile inoculating loop, aseptically place 2 or 3 loops of the culture** within this circle on the **top** of the slide (see Fig. 11). **Do not use water**.

   c. Using the inoculating loop, **spread the suspension over the area delineated by the circle** to form a thin film.

   d. Allow this thin suspension to **completely air dry**.

   e.To heat-fix the bacteria to the slide, **pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear near the opening of the microincinerator for 10 seconds** (see Fig. 6) as demonstrated by your instructor. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

In order to understand how staining works, it will be helpful to know a little about the physical and chemical nature of stains. Stains are generally salts in which one of the ions is colored. (A salt is a compound composed of a positively charged ion and a negatively charged ion.) For example, the dye methylene blue is actually the salt methylene blue chloride which will dissociate in water into a positively charged methylene blue ion which is blue in color and a negatively charged chloride ion which is colorless.

Dyes or stains may be divided into two groups: basic and acidic. If the color portion of the dye resides in the **positive ion**, as in the above case, it is called a **basic dye** (examples: methylene blue, crystal violet, safranin). If the color portion is in the **negatively charged ion**, it is called an **acidic dye** (examples: nigrosin, congo red).

Because of its chemical nature, **the cytoplasm of all bacterial cells has a slight negative charge** when growing in a medium of near neutral pH. Therefore, when using a **basic dye**, the positively charged color portion of the stain combines with the negatively charged bacterial cytoplasm (opposite charges attract) and the organism becomes **directly stained** (see Fig. 1). An **acidic dye**, due to its chemical nature, reacts differently. Since the color portion of the dye is on the negative ion, it will not readily combine with the negatively charged bacterial cytoplasm (like charges repel). Instead, it forms a **deposit around the organism**, leaving the organism itself colorless (see Fig. 1). Since the organism is seen indirectly, this type of staining is called **indirect** or **negative,** and is used to get a more accurate view of bacterial size, shapes, and arrangements.

In today's lab, we will make both direct and indirect stains of several microorganisms.

Concept map for Lab 5

B. DIRECT STAIN USING A BASIC DYE

In direct staining the positively charged color portion of the basic dye combines with the negatively charged bacterium and the organism becomes directly stained.

ORGANISMS

Your pure cultures of *Staphylococcus epidermidis* (coccus with staphylococcus arrangement) or *Micrococcus luteus* (coccus with a tetrad or a sarcina arrangement) and *Escherichia coli* (small bacillus) or *Enterobacter aerogenes* (small bacillus) from Lab 3.

- Pure culture of *Staphylococcus epidermidis*
- Pure culture of *Micrococcus luteus*
- Pure culture of *Escherichia coli*
- Pure culture of *Enterobacter aerogenes*

PROCEDURE (to be done individually)

1. *Escherichia coli* or *Enterobacter aerogenes*

   a. Heat-fix a smear of either *Escherichia coli* or *Enterobacter aerogenes* as follows:

1. Using the dropper bottle of **deionized water found in your staining rack**, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide (see Fig. 2). Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

2. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it 2 - 3 times to the drop of water** until the water **becomes visibly cloudy** (see Fig. 3).

3. **Incinerate the remaining bacteria on the inoculating loop.** If too much culture is added to the water, you will not see stained individual bacteria.

4. After the inoculating loop cools, **spread the suspension over approximately half of the slide** to form a thin film (see Fig. 4).

5. Allow this thin suspension to **completely air dry** (see Fig. 5). **The smear must be completely dry before the slide is heat fixed!**

6. To heat-fix the bacteria to the slide, **pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear near the opening of the microincinerator for 10 seconds** (see Fig. 6) as demonstrated by your instructor. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

b. Place the slide on a staining tray and cover the entire film with **safranin** (see Fig. 7A). Stain for **one minute**.

c. Pick up the slide by one end and hold it at an angle over the staining tray. Using the wash bottle on the bench top, gently **wash off the excess safranin** from the slide (see Fig. 7B). Also wash off any stain that got on the **bottom** of the slide as well.

d. Use a book of blotting paper to **blot the slide dry** (see Fig. 8). Observe using oil immersion microscopy.

Instructions for Focusing a Microscope from Lab 1.

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2. *Micrococcus luteus* or *Staphylococcus epidermidis*

a. Heat-fix a smear of either *Micrococcus luteus* or *Staphylococcus epidermidis*as follows:

1. Using the dropper bottle of **deionized water found in your staining rack**, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide (see Fig. 2). Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

2. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it 2 - 3 times to the drop of water** until the water **becomes visibly cloudy** (see Fig. 3).

3. **Incinerate the remaining bacteria on the inoculating loop.** If too much culture is added to the water, you will not see stained individual bacteria.

4. After the inoculating loop cools, **spread the suspension over approximately half of the slide** to form a thin film (see Fig. 4).

5. Allow this thin suspension to **completely air dry** (see Fig. 5). **The smear must be completely dry before the slide is heat fixed!**

6. To heat-fix the bacteria to the slide, **pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear near the opening of the microincinerator for 10 seconds** (see Fig. 6) as demonstrated by your instructor. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

b. Stain with **crystal violet** for **one minute**.

c. Wash off the excess crystal violet with water.

d. Blot dry and observe using oil immersion microscopy.

3. Prepare a third slide of the normal flora and cells of your mouth as follows:

a. Use a sterile cotton swab to vigorously scrape the inside of your mouth and gums.

b. Rub the swab over the slide (**do not use water**), **air dry**, and **heat-fix**.

c. Stain with **crystal violet** for **30 seconds.**

d. Wash off the excess crystal violet with water.

e. Blot dry and observe. Find epithelial cells using your 10X objective, center them in the field, and switch to oil immersion to observe your normal flora bacteria on and around your epithelial cells.

4. **Make sure you carefully pour the used dye in your staining tray into the waste dye collection container, not down the sink**.

Concept map for Lab 5

C. INDIRECT STAIN USING AN ACIDIC DYE In negative staining, the negatively charged color portion of the acidic dye is repelled by the negatively charged bacterial cell. Therefore the background will be stained and the cell will remain colorless.

ORGANISM

Use the pure culture of *Micrococcus luteus* provided.

PROCEDURE (to be done individually)

1. Place a **small drop of nigrosin** towards one end of a clean microscope slide.

2. Using your sterile inoculating loop, aseptically add a small amount of *Micrococcus luteus* to the dye and mix gently with the loop.

3. Using the edge of another slide, spread the mixture with varying pressure across the slide so that there are **alternating light and dark areas** (see Fig. 9). **Make sure the dye is not too thick or you will not see the bacteria**!

4. Let the slide **air dry completely** on the slide. **Do not heat fix and do not wash off the dye.**

5. Make sure you carefully pour the used dye in your staining tray into the waste dye collection container, not down the sink.

6. Observe using oil immersion microscopy. Find an area that has neither too much nor too little dye (an area that appears **light purple** where the light comes through the slide). If the dye is too thick, not enough light will pass through; if the dye is too thin, the background will be too light for sufficient contrast.

Concept map for Lab 5

RESULTS

Make drawings of your three direct stain preparations and your indirect stain preparation.

Direct stain of *Escherichia coli* or *Enterobacter aerogenes*

Direct stain of *Staphylococcus epidermidis* or *Micrococcus luteus*

Shape =	Shape =
	Arrangement =

Direct stain of epithelial cells and normal flora of your mouth

Indirect stain of *Micrococcus luteus*

Shape =

Arrangement =

PERFORMANCE OBJECTIVES FOR LAB 5

After completing this lab, the student will be able to perform the following objectives:

A. INTRODUCTION TO STAINING

1. Describe the procedure for heat fixation.
2. Define the following: acidic dye, basic dye, direct stain, and indirect stain.
3. State in chemical and physical terms the principle behind direct staining and the principle behind indirect staining.

B. DIRECT STAINING

PROCEDURE

1. Transfer a small number of bacteria from an agar surface or a broth culture to a glass slide and heat-fix the preparation.
2. Prepare a direct stain when given all the necessary materials.

RESULTS

1. Recognize a direct stain preparation when it is observed through a microscope and state the shape and arrangement of the organism.

C. INDIRECT STAINING

PROCEDURE

1. Perform an indirect stain when given all the necessary materials.
2. State why the dye is not washed off when doing an indirect stain.

RESULTS

1. Recognize an indirect stain preparation when it is observed through a microscope and state the shape and arrangement of the organism.

SELF-QUIZ

Self-quiz

Answers

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