Exercise 6-A
STAINING OF MICROORGANISMS
DIRECT VS INDIRECT STAINING

Introduction

The **morphological** features of individual microorganisms may be examined either by observing living, unstained materials, or by observing prepared slides containing killed organisms stained or colored with some type of dye. Observing dead, stained, microorganisms has certain advantages over observing living forms, as follows:

1. Staining increases the contrast between microorganisms (sometimes transparent) and their background, making them more readily visible.
2. Certain stains make cellular structures (internal or external) visible that otherwise cannot be seen.
3. Stained preparations are easier to observe under high magnification because dead cells do not swim, i.e., cannot exit the viewing field or focal plane.
4. The organisms present in stained preparations are dead, and if they were potentially pathogenic, are no longer capable of causing infection.

When a staining procedure colors the cells present in a preparation, but leaves the background colorless (appearing as white), it is called a **direct stain**. If a procedure colors the background, leaving the cells colorless (white) it is called an **indirect** or **negative stain**. Both direct and indirect stains will be used in this laboratory, and are sometimes used in combination. For example, the Gram stain, acid-fast stain, and Shaeffer-Fulton endospore stain are all direct stains. The capsule stains and Dorner method endospore stain are a combination of direct and indirect stains.

Stains or dyes are generally salts with one ion being colored and the other not. The colored ion is called a **chromophore**. If color is associated with the positive ion (**cation**), the stain is called a **basic stain**; if color is associated with the negative ion (**anion**), the stain is said to be **acidic**. Since cell membranes typically carry a slight negative charge, cells will readily attract and be colored by basic stains or dyes. M**ethylene blue**, **crystal violet** and **safranin** are examples of basic stains.

When preparing cellular materials for direct staining it is important to remember that morphological features are most readily visible if the cells present are separated from one another rather than being packed into a dense mass. One way to insure separation is to dilute the cellular material in liquid. A mixture of liquid (usually water) and microorganisms on the surface of a glass slide is called a **smear**, and is most useful if thin (similar to skim milk) and uniform in consistency. Students working in this laboratory are encouraged to keep their smears small in order to conserve both glass slides and staining reagents.

Prior to staining, it is necessary to air-dry and "**fix**" a smear, i.e., make the cells stick to the surface of a glass slide. One method used to fix cells to glass is called **heat-fixing**, and may be accomplished as outlined below. Heat-fixing will also kill the cells present in a smear, so will reduce the potential hazard of handling a slide containing pathogenic microorganisms.

The steps outlined below apply whenever cells are being prepared for direct staining; however, in this laboratory, they will most frequently be applied to bacteria.

Preparation of Cells for Direct Straining:

1. Use a clean glass slide and a sterile loop, and if transferring organisms from a solid surface (bacteria from agar or cheek epithelium) place a loopful of water on the slide first. Aseptically transfer the desired organisms into the water and mix to form the smear. If a broth culture is used, transfer several loopfuls to one spot, allowing each to air dry between applications.

2. Spread each smear to form a thin, uniform film and allow it to **air-dry** completely. **Do not use compressed air to dry a wet smear**! If smears are not allowed to dry thoroughly prior to heat-fixing, the cells present will be boiled and may take on unusual (abnormal) characteristics.

3. While holding one end of the slide, pass it **smear side up** through the flame of a Bunsen burner three times. **Use caution** – Too much heat will destroy the microorganisms and hot glass can cause burn injuries.

Application of Basic Stains

After smears have been prepared and heat fixed, they are ready for staining as indicated below. Since stain reagents are potentially messy and some can cause skin irritation, students are encouraged to use caution and to avoid unnecessary contact between stain reagents and skin surfaces.

1. Place the heat-fixed slide, **smear side up**, on a slide rack over a sink**.** Cover each smear with one or more drops of the appropriate stain reagent and allow the stain to act for the required time (60 seconds for methylene blue and safranin; 10 seconds for crystal violet). **DO NOT OVER STAIN!**

2. Remove the slide from the stain rack, hold it at an angle toward the bottom of the sink and rinse the stained smear thoroughly with tap water.

3. Remove excess water from the slide bottom with paper towel and air-dry the smear surface. Compressed air may be used to "push" excess water from the slide, but be prepared to catch the displaced liquid in a paper towel. Blotting the preparation with a Kimwipe will also speed drying, but can damage the smear. Dried smears are ready for examination.

Indirect or Negative Staining with Acidic Stains

Stains or dyes composed of neutral particles, or with colored anions, are not attracted to the negatively charged surfaces of cells. These stains do not color cells, but will instead form a deposit around and between cells, making them visible indirectly as colorless objects against a dark background. Although indirect or negative staining is not used extensively in this laboratory, it does have an advantage over direct staining because it causes less cellular distortion. The procedures used for indirect or negative staining do not require smears to be heat-fixed prior to stain application, so cells retain their actual size and shape. **Nigrosin**, an aqueous solution containing carbon particles, and **Congo red,** an acidic stain, can both be used for indirect or negative staining procedures as follows:

1. Place one or two loopfuls of water on the surface of a clean glass slide and then using a cooled, sterile loop, aseptically add enough cellular material to form a smear with the consistency of thin skim milk (not pea soup).

2. Aseptically add a loopful of stain reagent (either nigrosin or congo red) to the smear, mix gently and then spread the smear over the glass surface forming a thin film. A properly prepared nigrosin smear will appear dark gray, while a congo red smear will appear coppery-red.

3. Allow the smear to air dry and gently heat fix. If nigrosin was used, the preparation is complete at this point. If Congo red was used, proceed to step 4-5 below.

4. Flood the Congo red smear with acid-alcohol and allow it to stand for 60 seconds. Exposure to acid causes the Congo red to darken (it usually turns blue).

5. Gently rinse the acid-alcohol from the Congo red preparation with clean tap water, remove excess water from the slide bottom and then allow the smear to air dry.

Note - Do not allow water to contact smears prepared with nigrosin because water will rinse the nigrosin from the slide and ruin the preparation. **Remember** that non-heat-fixed smears probably contain viable cells and must be disposed of in the proper manner.

Procedure:

1. Place a loopful of water on the surface of a clean glass slide, and then using a clean toothpick, gently scrape some dead, squamous epithelial cells from the inner surface of your cheek. Be careful not to injure any soft, living tissue.

2. Form a smear by mixing the cells in the water on the slide. Spread the smear to form a thin film, allow it to air dry and heat-fix it.

3. Stain the epithelial cells with one of the direct stains provided as indicated above (60 seconds for methylene blue/safranin or 10 seconds for crystal violet), and then rinse the preparation with water and allow it to air dry.

4. On a new slide, prepare a negative stain of your morphological unknown culture using either nigrosin or Congo red. **Note** – If the culture has not yet formed colonies, a smear can be made from the original broth by collecting cells from the tube bottom (do not add water).

5. Air dry and gently heat-fix all negative stain preparations made with nigrosin to insure the cells present are dead. Apply acid-alcohol to all Congo red smears, rinse with tap water and dry.

6. Use the compound microscope to examine each preparation. **Note** - Although bacteria are small (typically about 1μm in diameter) and can only be viewed properly with the oil immersion lens, remember to begin focusing using the low power (10X) objective.

7. Measure (in microns) one or more of the cheek epithelium (eukaryotic) cells present while viewing the preparation with the 10X lens. Look for eukaryotic cell structures such as the cell membrane, nucleus, nuclear membrane and cytoplasm.

8. Record your observations as indicated on the worksheet provided. Be careful to include measurements and labels for eukaryotic structures observed.

9. Add a drop of immersion oil to your cheek cell preparation and examine it using the 100X lens. Look for bacteria on the eukaryotic cell surfaces, and record their size and shape.

10. Observe your indirect stain preparation with your 100X lens (be sure to focus with the 10X lens first, and don't forget to add immersion oil).

11. Determine the shape, size (in microns) and arrangement of the cells present in your morphological unknown culture.

12. Record this information on the worksheet provided along with an accurate representation of what the cells look like.

Note - You will ultimately be required to turn in illustrations of your morphological unknown culture as it appears in a variety of stain preparations, so it is advisable to make your worksheet illustrations as accurate as possible. When illustrated for the morphological unknown report, the cells must be magnified 5000X, so do not draw them as dots or specks.

All organisms used for morphological unknown cultures are as large or larger than those shown in the section on bacterial morphology (Fig. 6.1). Make your illustrations accordingly.

Questions:

1. How do direct stains differ from indirect stains in terms of ionic composition (the charge associated with their chromophore) and their interactions with cell surfaces, i.e., how they work?

2. Why is it important to form bacterial smears by mixing cells with a small amount of liquid and spreading them over a slide surface?

3. What are two functions of heat-fixing bacterial smears prior to staining?

4. What is the approximate size relationship between the prokaryotic and eukaryotic cells found in a cheek cell preparation?

5. Were all of the cells associated with your morphological unknown the same shape? Were they all the same size? Was their arrangement consistent?

Exercise 6-A-Supplement
BACTERIAL MORPHOLOGY

Introduction

Individual bacteria typically have one of three shapes; spherical (**cocci** - singular **coccus**), rod-like or cylindrical (**bacilli** - singular **bacillus**), or spiral (**spirilla** - singular **spirillum**). Depending upon the manner in which they divide, bacteria may occur as single cells or as multiple cells in specific arrangements as shown.

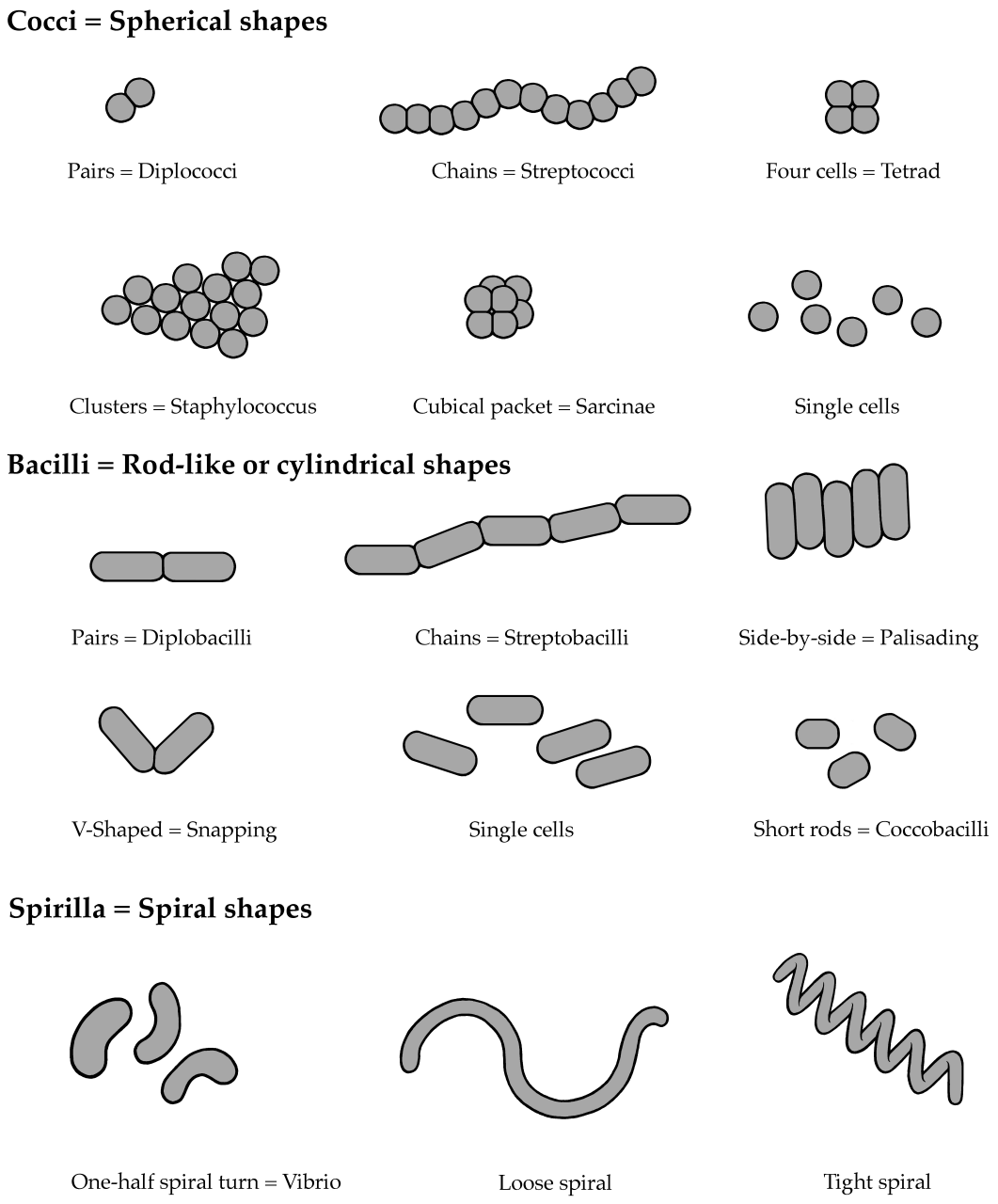


Fig. 6.1 - Some examples of bacterial cell morphology and arrangement

Cocci may occur singly, or can assume a wide variety of arrangements as follows:

1. Pairs of cells = **diplococci**
2. Chains of four or more cells = **streptococci**
3. Four cocci in a box-like or square arrangement = **tetrad**
4. Cubical packets of eight cells each = **sarcinae** (singular **sarcina**)
5. Irregular grape-like clusters of cells = **staphylococci**

Bacilli may also occur in pairs - diplobacilli, or in chains - streptobacilli. Some cells form short rounded rods that are hard to distinguish from cocci. These are termed cocco-bacilli. Rods arranged in a pattern resembling a picket fence are said to be palisading.

Spiral-shaped bacteria exhibit differences in their spiral shape and rigidity. The term **vibrio** refers to a comma-shaped cell, or one-half a spiral turn**.** Bacteria known as **spirochetes** form thin, flexible, spiral-shaped cells. The cyanobacteria called *Spirulina* form long slender spirilla that often wrap around one another forming complex net-like arrangements.

Many forms of bacteria do not fit nicely into the categories listed above. Some occur as rods (bacilli) of irregular shape, some are pleomorphic (able to change their shape) and some are branching. Bacteria in the genus *Streptomyces* typically form filamentous cells resembling the hyphae of mold-type fungi. These long, slender thread-like cells usually have numerous branches that sometimes terminate in a string of cocci-shaped conidia (reproductive cells). A few types of bacteria are even more unusual, occurring as squares, stars or triangles. Most of the bacteria we will be working with in this laboratory occur as cocci or bacilli (rods).

Name_____ **Lab Section**_____

WORKSHEET
Exercise 6A
Staining of Microorganisms: Direct vs. Indirect Staining

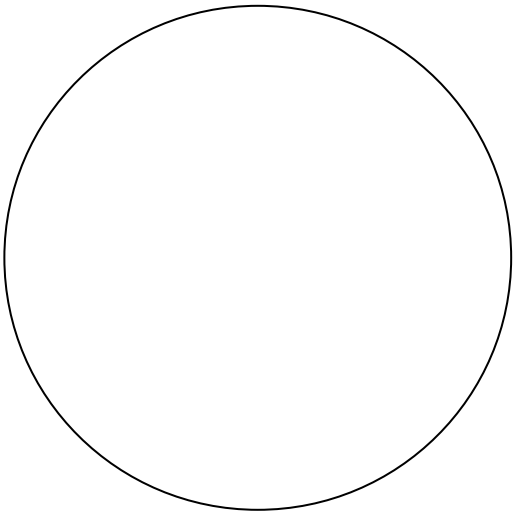
Goals: _____
_____

Materials & Methods:

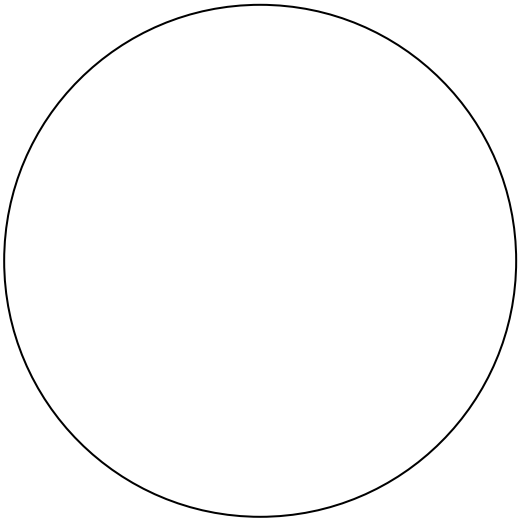
1. Direct Staining
   Date of smear: _____ Specimen: _____
   Stain used: _____ Duration of stain: _____
2. Indirect Staining
   Date of smear: _____ Specimen: _____
   Stain used: _____ Age of specimen: _____

Data & Results:

1. Direct Staining

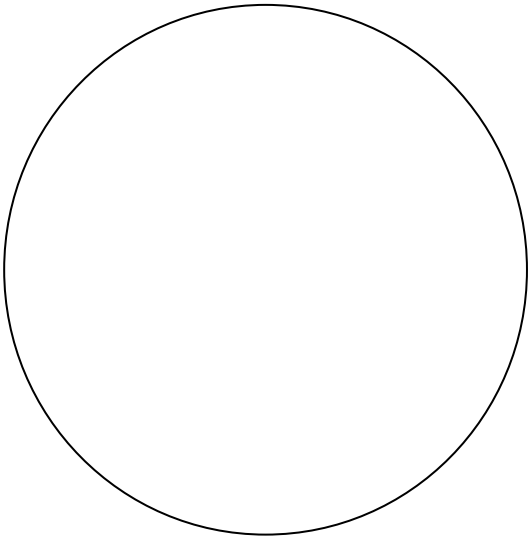


Specimen: Cheek cell smear – Cheek Cells			
Total Magnification:			
Length:	units x	μm/unit =	μm
Width:	units x	μm/unit =	μm
Notes:			



Specimen: Cheek cell smear - bacteria			
Total Magnification:			
Length:	units x	μm/unit =	μm
Width:	units x	μm/unit =	μm
Notes:			

2. Indirect Staining



Specimen: Morphological Unknown			
Total Magnification:			
Length:	units x	μm/unit =	μm
Width:	units x	μm/unit =	μm
Notes:			

Conclusions:

Part 1 – Direct Stain

Based on your data, what can you conclude about your oral cavity? _____

_____

_____

Based on your data, compare the sizes of prokaryote and eukaryote cells. _____

_____

_____

Part 2 – Indirect Stain

Describe the cell morphology of your Morphological Unknown. _____

_____

_____