Lab Exercise 5: The Smear and Simple Staining

Background
Smears
The preparation of a good smear is the backbone of all of the staining techniques performed throughout this course. A good smear is the result of the mastery of three individual concepts:
1. Heat fixing/adhering the cells to the slide so that they are not washed off during subsequent staining procedures.
2. Heat fixing gently so as not to cause distortion of the cells.
3. Preparing a thin smear, because the thickness will determine whether or not you can visualize individual cells, their arrangement or details regarding Gram reaction or internal structure.
4. Preparing a smear that isn't too thin, so organisms have actually been transferred to the slide.

Simple staining
The use of a single stain or dye to color a bacterium is called a simple stain (figure above). During the simple staining technique, a single dye is used to color the otherwise colorless bacterial cells. These types of dyes, called basic or positive dyes, are positively charged, containing cationic chromophores. Because all cells are negatively charged (i.e., -70mV), there is an attraction between the positive charge of the dye and the negative charge of the cell. Some common basic dyes used in staining are methylene blue, crystal violet and basic fuchsin. However, not all dyes are positively charged. Acidic or negative dyes are negatively charged, containing anionic chromophores, and are actually repelled by the negatively charged bacterial cells. They are usually used for negative staining protocols which are the subject of a later lab. Common acidic dyes are nigrosin, India ink and eosin.

Simple stain of Escherichia coli using the basic/positive dye Methylene Blue. Note the single bacilli which are characteristic of E. coli. 1000×

Staining microbial cells is important because without stain, most bacterial cells are extremely difficult to see. Staining allows them to be seen so that observations as to their morphology (i.e., individual cell shape) and arrangement (i.e., how the cells remain physically attached to one another after cell division) can be made. Bacteria are morphologically categorized as either cocci (singular: coccus) if they are round; bacilli (singular: bacillus) if they are rod-shaped; or spirilla (singular: spirillum) if they are spiral-shaped. Although there are other bacterial morphologies, including the characteristic corkscrew-shaped spirochetes, the three mentioned above are by far the most common. As such, you should be able to identify, describe and correctly name cocci, bacilli and spirilla.

In addition, bacteria are characterized based on the arrangement of cells. Cells that are arranged in pairs are described with the prefix diplo-, cells that are arranged in chains are strepto- and cells arranged in clusters are staphylo- in arrangement. It is important to note that morphology and arrangement are determined microscopically and must be identified in areas of your field of view where the cells are not crowded together because of a smear that is too thick. Overcrowding should not be confused with true staphylo- arrangements.

These arrangements are the result of bacterial reproduction or binary fission (figure below). When fission of the cell is incomplete, due to the genetic programming of the cell, cells may remain physically attached to one another, causing the aforementioned bacterial arrangements. Tetrad and staphylo- arrangements of cells result from binary fission along more than one plane of division, and are only possible when cocci reproduce. For this reason, you will never see bacilli or spirilla arranged in tetrads or clusters.
Introduction
Today, the *Escherichia coli* cultures from the Aseptic Technique lab will be stained. These cultures will be examined for morphology and arrangement indicative of *E. coli* and look for the presence of additional microbial contaminants. Because these are pure cultures that should have been aseptically transferred, there should only be one bacterial species on each of the slides prepared today and all of them should be identical in size, morphology and arrangement. Unless otherwise noted, the smear preparation technique that is being introduced today will be used every time a slide is prepared for microscopic examination. As such, care must be taken to master this technique.

Objectives
1. Prepare bacterial smears for the microscopic visualization of bacteria.
2. Perform the simple staining procedure.
3. Confirm the aseptic transfer of *E. coli* from all for the Aseptic Technique lab broths and agar slants.

Smear Preparation Protocol

<table>
<thead>
<tr>
<th>Individual supplies</th>
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<tr>
<td><em>E. coli</em> slants &amp; broths from the Aseptic Technique lab exercise (4 total)</td>
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<tr>
<td>Glass microscope slides &amp; KimWipes</td>
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<tr>
<td>Inoculating loop</td>
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<tr>
<td>Water bottle</td>
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1. Prepare the lab bench by removing extraneous items and cleaning the surface with table disinfectant.
2. Wipe a microscope slide clean with a paper towel or KimWipe.
3. Using a grease pencil, draw a circle about the size of a dime on the bottom of the slide.
4. Transfer culture to the TOP of the slide, within the circle previously drawn.

From the solid culture:
1. Place one drop of water from the loop onto the center of the circle
2. Transfer a small amount of solid inoculum into the drop of water and disperse it thoroughly.
3. Spread the liquid into a thin area about the size of a dime.
4. Allow the smear to air dry.

From the liquid culture:
1. Place 3–4 loopfuls of liquid culture on the center of the circle.
2. Spread the suspension into a thin area about the size of a dime.
3. Allow the smear to air dry. Note: if the inoculation on the slide is completely transparent after drying, add a few more loopfuls of organism.
4. Regardless of the culture source, when the smear is completely dry, heat-fix by quickly passing the smear over the flame two to three times.
5. Repeat this smear prep procedure for the remaining three cultures.
Simple Stain Protocol

<table>
<thead>
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<th>Individual Supplies</th>
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<tr>
<td>Heat-fixed slides (from the Smear Preparation Protocol)</td>
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<tr>
<td>Methylene blue dye</td>
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<tr>
<td>Bibulous paper</td>
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<tr>
<td>Staining tray</td>
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<td>Water bottle</td>
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1. Place the slide on the staining tray and cover the smear with Methylene Blue dye for approximately 1 minute.

2. While holding the slide at a 45° angle, gently wash the surface with water 2–3 times. Allow the wash to drain into the staining tray.

3. Open the package of bibulous paper and place the wet slide inside. Close the package and carefully press on the cover to blot the slide dry. Do not blot too vigorously or the slide will crack. There is no need to remove paper from the bibulous paper pad.

4. Once the slides are dry, examine them microscopically. Be sure to use the 100× objective and oil.

Microscope Use Protocol

Viewing Your Specimen

1. Plug in the microscope, taking care to insure the cord is firmly attached to its back.
2. Turn on the power switch. Increase the brightness control dial until it is at its maximum position.
3. Place a microscope slide on the stage and secure it in place against the corner of the stage using the specimen holder (4) (the slide should sit against this arm, not under it). Center the slide over the light source using the longitudinal (9) and latitudinal (10) stage motion knobs.
4. Rotate the revolving nosepiece until the 4× objective lens is locked in place over the slide. Make sure that the condenser aperture diaphragm lever is set to 4. Raise the stage to its top-most position.
5. While looking through the eyepieces, adjust for your interpupillary distance by distancing the eyepieces until you can see one image.
6. While still looking through the ocular lenses, slowly lower the stage using the course adjustment knob until the specimen comes into focus. If you are unsure whether the object you are viewing is on your slide, very slightly move the stage back and forth while looking at it.
7. Once you have the specimen in coarse focus, use the fine focus knob to bring the specimen into sharp focus. Using the X and Y stage motion knobs, center the object in your field of view. Note: If you have markedly different eyesight between your eyes, close one eye and adjust the fine focus until the object is in sharp focus. Close the other eye and adjust the diopter ring until the object is again in sharp focus.
8. Rotate the revolving nosepiece to the 10× objective. Adjust the condenser aperture diaphragm lever to 10. Bring the specimen into the center of your field of view. Once you have the specimen in coarse focus, use the fine focus knob to...
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9. Rotate the revolving nosepiece to center the 40× objective. Adjust the condenser aperture lever to 40. Bring the specimen into the center of your field of view. Once you have the specimen in coarse focus, use the fine focus knob to bring the specimen into sharp focus. Note: Because the 40× objective has a small opening, it is often difficult for adequate light to enter the objective. This sometimes results in blurry and dark images that may not be correctable.

10. Prepare to view the specimen using the 100× objective by rotating the revolving nosepiece so that the space between the 40× and 100× objective is directly over your slide.

11. Add one drop of immersion oil directly to the slide.

12. Slowly move the 100× lens into place. The 100× lens should be fully immersed in the oil.

13. Using the fine focus knob, bring the object into sharp focus. Note: Once you have added oil to the slide, you can no longer use your 40× objective. If you cannot find your specimen, you must rotate to 10× only. Do not allow the 40× to pass by the slide again or it will be contaminated with oil.

Cleaning and Putting Away Your Microscope

1. Using the brightness control dial, turn down the light completely.
2. Turn off the microscope.
3. Unplug and wrap the cord around the microscope.
4. Using the coarse focus knob, lower the stage completely.
5. Using LENS PAPER and LENS CLEANER clean the objective lenses. There should only be oil on the 100× objective, but confirm that there is no oil on any of the objectives or the stage. In order to remove all of the oil, apply GENTLE pressure to the bottom of the 100× objective to allow the trapped oil to drain out.
6. Turn the revolving nosepiece so that the 4× objective is directly above the stage. Using the longitudinal and latitudinal stage motion knobs, move the stage in a position close to the arm.
7. Carefully place the microscope in its designated cupboard space with the oculars facing into the cupboard.
8. Lay the cord on the hook at the back of the microscope.

Data Collection and Analysis

1. Draw observations of the slide preparations, taking care to indicate total magnification. Make sure to properly describe the morphology and arrangement of the cells as they are observed.

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<tr>
<th>Specimen</th>
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<td><img src="image1.png" alt="Specimen" /></td>
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Total magnification _____× | Total magnification _____×
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**Discussion**

1. How does smear preparation of cells from a liquid medium differ from preparation of cells from a solid medium?
2. Why is it important to limit the quantity of cells used to prepare a smear?
3. For preparation of a smear on a slide, what is the purpose of heat fixation? What problems can arise when the slide is heated in a flame?
4. What causes a stain to adhere to bacterial cells? Why are all colored dyes not necessarily useful for simple staining?
5. How are both basic/positive and acidic/negative stains used in staining protocols?

Don’t forget to complete the Data Collection & Analysis and Discussion sections of the Aseptic Technique lab.