Microscopy Lab II

Aim

To perform gram staining of provided bacterial cultures and visualize under the compound microscope

Background: The Gram stain is used to differentiate between gram positive organisms and gram negative organisms. Hence, it is a *differential* stain.

Gram staining involves a four-part process, which includes:

crystal violet, the primary stain
iodine, the mordant
a decolorizer made of acetone and alcohol
safranin, the counterstain

Gram negative and gram positive organisms are distinguished from each other by differences in their cell walls. These differences affect many aspects of the cell, including the way the cell takes up and retains stains.

Gram positive cells take up the crystal violet, which is then fixed in the cell with the iodine mordant. This forms a crystal-violet iodine complex which remains in the cell even after decolorizing. It is thought that this happens because the cell walls of gram positive organisms include a thick layer of protein-sugar complexes called peptidoglycans. This layer makes up 60-90% of the gram positive cell wall. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. At the end of the gram staining procedure, gram positive cells will be stained a purplish-blue color.

Gram negative cells also take up crystal violet, and the iodine forms a crystal violet-iodine complex in the cells as it did in the gram positive cells. However, the cell walls of gram negative organisms do not retain this complex when decolorized. Peptidoglycans are present in the cell walls of gram negative organisms, but they only comprise 10-20% of the cell wall. Gram negative cells also have an outer layer which gram positive organisms do not have; this layer is made up of lipids, polysaccharides, and proteins. Exposing gram negative cells to the decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. This allows the cells to subsequently be stained with safranin. At the end of the gram staining procedure, gram negative cells will be stained a reddish-pink color. (http://www.austincc.edu/microbugz/gram_stain.php)

Points to consider before starting experiment with microscopes:

Cleaning of mechanical parts, lenses etc.

Carrying microscope: Grasp by its arm only if highly required. Put a hand at the bottom for support

Observation with scanner, low-power, and high-dry objectives:

1. As we will be using **binocular microscopes**, you have to adjust two eyepiecesas per your level of comfort in such a way that the two fields of views should converge to give you one field of view.
2. Place the slide on the stage. Use the knobs of the mechanical stage to move the slide, centering the object in the hole in the center of the stage.
3. Select the scanner (4X) objective. You should begin all microscopic observations on low power. This is the only way to properly focus the microscope. Grasp the nosepiece (not the objectives) and rotate it until the low power objective clicks into place. (If your microscope does not have a scanner lens, then begin with the low-power lens.)
4. Focus the microscope on the scanner power. With the coarse focus knob, raise the stage until it stops. With the fine focus, bring the object into sharp focus. You should be able to focus with only a slight movement of the fine focus.
5. Observe the slide under the scanner power. Use the mechanical stage knobs to find the object and center it in the field of view.
6. Set the proper illumination. Adjust the iris diaphragm until you can see the maximum amount of detail. Use the minimum amount of light necessary to get the best contrast and resolution.

7. Switch from scanner to low-power (10X) objective. After you have focused the object on the scanner, rotate the low- power lens into place. Do not change the focus knobs before moving the nosepiece. Microscopes used in microbiology are **parfocal**, which means that they keep their focus from one objective lens to the next. Once you have moved the low-power lens into place, bring the object into sharp focus with the fine focus knob. Use the iris control to set the best illumination.
8. Switch from low-power to high-dry. After you have focused the object on the low- power, rotate the high-dry lens into place. Do not change the focus knobs before moving the nosepiece. Once you have moved the high-dry lens into place, bring the object into sharp focus with the fine focus knob. Use the iris control to set the best illumination.

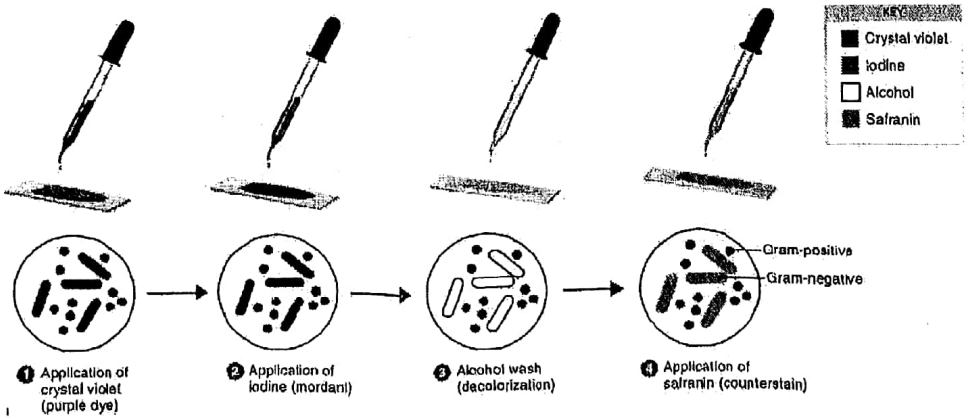
Using the oil immersion lens: (This section you will need to know for your next class to visualize slides of stained bacteria)

1. First, you must focus the microscope using the scanner, low power, and high-dry objectives.
2. With the microscope sharply focused with the high-dry lens, rotate the nosepiece halfway to the oil immersion objective, and place a small drop of immersion oil directly on the specimen. Continue to rotate the nosepiece until the oil immersion objective clicks into place. The oil should touch the bottom of the lens and fill the gap between the lens and the object. Focus with the fine focus only until the object comes into view.
3. Never use immersion oil with any lens other than the oil immersion lens. The low-power and high-dry lenses do not have the proper gaskets, so oil can seep into the objective and cause it to become permanently blurred.
4. Clean the oil immersion lens after each use, using the technique demonstrated by the instructor. You will use the oil immersion lens, the objective with the highest magnification, for most of your work. It is 100X on most microscopes, and it works properly only when used with immersion oil. The oil acts to reduce the amount of light lost by scattering, or refraction. This greatly improves the resolution at the highest magnification.

Preparing specimen slides for observations:

Gram staining for bacteria:

1. Transfer a drop of the suspended culture to be examined on a slide with an inoculation loop/micropipette.
2. Spread the culture with an inoculation loop to an even thin film over a circle of 1-1.5 cm in diameter.
3. Air-dry the culture and fix it,
4. Add crystal violet stain over the fixed culture. Let stand for 30 to 60 seconds;
5. Pour off the stain and gently rinse the excess stain with a stream of water from a faucet or a plastic water bottle.
6. Add the iodine solution on the smear, just enough to cover the fixed culture. Let stand for 30 seconds. Pour off the iodine solution and rinse the slide with running water. Shake off the excess water from the surface.
7. Add a few drops of decolorizer so the solution trickles down the slide. **Rinse it off with water after 5 seconds. Counter stain with basic fuchsin/safranine solution for 40 to 60 seconds.**
8. Wash off the solution with water. Blot with paper to remove the excess water.
9. View the smear using a light-microscope under oil-immersion after adding a very small drop of oil.



http://www.medicinehack.com/2012/02/gram-staining-procedure-mechanism.html