Brightfield microscopy allows magnification of images by using two different lens systems, the **ocular lens** and the **objective lens**. The objective lenses are located on a revolving nosepiece, with usually 3 to 4 found on each microscope. Most commonly, these lenses provide magnifications of 10X (low power), 40X (high-dry), and 100X (oil immersion). The ocular lens, or eyepiece, is found at the top of the microscope and usually has a magnification of 10X. Microscopes that have one ocular lens are referred to as monocular, and those having two ocular lenses are called binocular. A third important lens is the condenser lens found below the stage of the microscope. The condenser lens focuses the light on the specimen being studied. The substage adjustment knob controls the movement of the condenser lens. When using the 100X oil immersion objective, make sure that the condenser lens is in the uppermost position just under the stage in order to allow for maximum light capture. An iris diaphragm regulates the amount of light passing through the condenser lens from the tungsten bulb in the base of the microscope. A blue filter is usually positioned above the light source in order to improve resolution. Magnification of the image is determined by multiplying the power of the ocular lens by the power of the objective lens. The maximum magnification using the 100X oil immersion objective is 1000X when using the normal 10X ocular lens. Most microscopes are also equipped with a mechanical stage to allow for quick positioning of the specimen. Locate the controls for the mechanical stage, and become familiar with the directions of movement possible.

The resolving power is the ability to distinguish two images as separate. The resolving power depends on the wavelength of radiation used and the numerical aperture of the system. Resolving power = wavelength/(2xN.A). The maximum resolving power of the best light microscope is 0.2 um. Remember this when trying to distinguish the morphologies of some of the tiny “dots” of bacteria on the slides. The numerical aperture describes the light-gathering capabilities of a particular lens system being used, and is influenced by the refractive index of the lens; that is, it is a measure of the deviation or bending of light rays as they pass from one medium to another. The deviation occurs at the surface of the junction of the two media, which is known as the refracting surface.

The refractive index for air is 1.00. The refractive index for glass is 1.25. In order to prevent the bending of light rays as they are passing from air into the glass lens, immersion oil is placed directly on specimens to be examined with the 100X objective. The refractive index of the immersion oil is the same as for glass, so the path of light rays is not bent when moving from one medium to another. One unfortunate aspect of using oil is the potential damage that can be done to the 100X objective lens if the oil is not properly removed from the lenses after use. Therefore, **remember to thoroughly clean the oil objective after each use**.
A wet mount enables microbes to be observed in their natural (live) state, so that characteristics such as motility, morphology, and intercellular organization can be observed. Simple staining procedures use a single stain to help visualize the individual cells of microorganisms as deposited on a slide using a smear-preparation technique. Most dyes used in this procedure (crystal violet, methylene blue, or basic fuchsin) contain chromophoric (colored) cations (positively charged ions). Since the surfaces of bacterial cells have a slightly negative charge, the positively charged dyes have an affinity for the cells. The dyes that interact in this fashion with the bacterial cells are referred to as basic dyes. Other dyes that are anionic, that is, those in which the chromatophore carries a negative charge, are useful in other staining techniques.

The Gram stain was developed in 1884 by Hans Christian Gram, a Dutch bacteriologist, to help study a group of spherically shaped bacteria isolated from human lung tissue. The Gram stain is used to differentiate types of bacteria depending on their ability to retain a particular stain. It is therefore referred to as a differential staining technique.

The Gram stain is one of the most frequently used techniques in microbiology, and its mastery can be difficult. In order to become proficient, pay careful attention to the procedure and practice it often. The technique separates bacteria into two groups: gram-positive and gram-negative. The first step of the Gram stain involves staining the fixed smear of organisms with a primary stain of crystal violet. This is followed by the application of Gram’s iodine stain, also known as the mordant (a substance capable of intensifying or deepening the reaction of the specimen to a stain). The next step in the procedure is the most critical and involves washing the stained smear with a decolorizing agent, usually 95% ethanol or isopropyl alcohol. The last step employs a counterstain known as safranin. Gram-positive organisms will not be easily decolorized and thus retain the purple stain of crystal violet. On the other hand, the gram-negative organisms will be decolorized by the alcohol so that they then can take up the counterstain. They will appear pinkish or red. If enough alcohol is applied, it is possible to decolorize almost all cells. Careful adherence to the procedure will help to ensure successful results.

The age of the culture is an important factor in the outcome of the Gram stain. Most gram-positive organisms will lose their gram-positivity with age. The Gram stain should always be performed on vigorous, actively growing cultures (18- to 24-hour cultures usually will give excellent results). To further complicate interpretation of results, some organisms turn out to be gram-variable following Gram staining. This means that some of the cells in the population will stain purple (gram-positive) and other cells will stain red (gram-negative). Only repeated staining of the same culture at different times will verify the existence of a truly gram-variable culture. Gram-variability is relatively rare; mixed results in the Gram stain most probably occur from working with an impure culture, or either under-decolorizing or over-decolorizing with ethanol.

The basis of the Gram stain resides in the differences in cell wall composition of gram-positive and gram-negative bacteria. Be sure to read your textbook for a complete discussion of the topic. Briefly, gram-positive organisms have a thick cell wall composed of teichoic acid and peptidoglycan, a polymer of sugars and amino acids. On the other hand, gram-negative organisms have a much thinner cell wall and have an outer membrane composed of lipopolysaccharide and lipoprotein. Two mechanisms have been proposed to explain the basis of the Gram reaction. One mechanism advanced is that the alcohol decolorizer extracts lipid from the outer membrane of gram-negative bacteria, thus facilitating the loss of the crystal-violet complex. A second mechanism proposed is the shrinkage of pores in the thick peptidoglycan layer of cell walls of gram-positive organisms due to exposure to the alcohol decolorizer. The shrinkage of the pores leads to retention of the dye complex in gram-positive cells; whereas, the higher porosity of the thin peptidoglycan layer of gram-negative organisms leads to more rapid loss of the dye complex. Differences in cell wall composition of gram-positive and gram-negative organisms also account for the differences in susceptibility to various antibiotics.
A. Prepare smears of known and unknown bacteria to be used for Gram Stain.

1. Always begin work by disinfecting your work area. Spray disinfectant on the benchtop and wipe off with paper towels.

2. Label the end of 2 slides each (4 total) as follows – UK-XXX, K-XXX, where XXX are your initials

3. Place a drop of water (perhaps pipette 5 uL onto the slide) onto the center of each slide, use an inoculating needle to pick up a small amount of bacteria from a single colony and spread it in a small drop of water. If the smear is too dense (i.e., contains a large number of organisms), staining and differentiating the organisms will be difficult. Make sure to spread out the liquid on the surface of the slide to facilitate drying and distribution of the organisms.

4. Allow the smears to dry at room temperature or on the slide warmer. Do not blow on the slide. Remember, we are laden with “germs” and are not interested at this point in determining the types of microbes in human breath! It is a good idea to prepare and observe wet mount (see part D) while air drying.

5. Gently heat-fix the slides by passing the dried smears through the flame of the Bunsen burner. Caution must be exercised not to overheat the slide. Remember to gently heat-fix and not cook the bacteria on the slide.

B. Prepare Gram Stain

1. Place the known and unknown fixed smear slides on a staining tray rack and cover the smear with the crystal-violet stain. Allow the stain to react for 1 min.

2. Using the wash bottle, rinse the slide with a small amount of water. Make sure to remove excess water by shaking the slide.

3. Cover the smear with the Gram’s iodine stain and allow to react for 1 min. Wash off the stain with water.

4. Holding the slide at a 45” angle over the sink or staining tray, apply the decolorizer (95% ethanol) dropwise at the top of the slide and allow the alcohol to run off the slide for 2-3 seconds. Immediately rinse the slide with water. Remove the excess water by shaking.

5. Counterstain by flooding the smear with safranin for 1 min.

6. Rinse with water and blot dry by placing between pages of the bibulous pad. Wipe the bottom of the slide to remove excess stain that may decrease visibility.

Critical precautions to take when performing Gram stains:
- Do not overdecolorize slides with alcohol.
- Use actively growing cultures.
- Prepare thin smears containing a sparse number of organisms.
- Do not overheat the slide during the fixation step as structural morphologies of the cells may be altered: Overheating during the fixation step may influence the structure of the cell wall and, as a result, alter the outcome of the stain.
C. Observe slides

1. Carefully remove a microscope from the storage cabinet and position it in a clear area on the lab bench directly in front of you.

2. Take a few moments to familiarize yourself with the location of the various parts of the microscope. If you have difficulty locating a part or are unclear regarding its use, ask your lab instructor for help before proceeding.

3. Be sure the microscope is plugged in and that the lamp has been turned on.

4. Position yourself at a height so that you can comfortably make observations through the ocular lenses without straining your neck or back.

5. Be sure that the microscope remains flat on the lab bench at all times. Do not tilt your microscope when you are observing specimens in this laboratory.

6. Clean all glass surfaces with lens paper.

7. Raise the condenser lens to its highest position beneath the stage of the microscope. Also check to verify that the iris diaphragm is open.

8. Turn the coarse adjustment knob until there is an ample working distance between the objective lenses and the stage of the microscope.

9. Place a slide on the stage of the microscope using the specimen holder clamp. Try to position the slide so that the stained smear is in the viewing area.

10. Swing the low-power (10X) objective into place by revolving the turret which holds the objective lenses. Be sure that the lens is locked into position.

11. While looking from the side, turn the coarse adjustment knob until the 10X objective is positioned as close as possible to the slide. Most microscopes have stops that prevent the objective lenses from touching the slides on the stage of the microscope. If the microscope does not have the safety stop feature, be sure the condenser lens does not touch the slide.

12. Look through the ocular lens and slowly increase the distance between the lens and the slide. When an image comes into focus, stop and adjust the amount of light by regulating the iris diaphragm. Use the fine adjustment knob for critically focusing the specimen. When using a binocular microscope, be sure to position eyepieces so that both eyes are kept open. Using both eyes will significantly reduce eye strain.

13. Move the slide by using the knobs of the mechanical stage to focus on the air-water interface of a bubble.

14. Next, rotate the turret and swing into place a higher-powered objective (40X). Observe the specimen. It should still be in focus. Most microscopes are parfocal, which means that when a specimen is in focus using one lens, it remains in focus when switching to another objective lens. A slight adjustment of the fine adjustment knob may be necessary to bring the specimen into sharp focus.
15. Gram stains should always be viewed with the oil immersion lens. To use this lens, rotate the turret halfway between the 40X and 100X objectives, place a drop of immersion oil onto the specimen and rotate the 100X objective into position. Check to be sure that the oil is contacting both the lens and the slide.

16. Use the fine adjustment focus knob for critical focusing. Make sure that your condenser lens is in the uppermost position under the stage. If objects are not visible, be sure to check that the iris diaphragm is open. Reducing the amount of light illuminating the specimen will enable a better description of the cellular morphologies. Use the mechanical stage to observe different areas of the slide.

17. Record the Gram stain reaction of each of the organisms, the morphology of the individual cells (i.e., rod, spherical, or spiral shaped), and the arrangement of the cells (clusters, chains, or pairs). Use a digital camera to document the appearance of your organisms’ gram stains.

18. Always conclude work by disinfecting your work area. Spray disinfectant on the benchtop and wipe off with paper towels.

D. Prepare wet mounts of known and unknown bacteria.

1. Place 20 uL of suspended liquid cultures (preferred) onto a clean slide or inoculate from agar culture with a needle into a drop of water on the slide. Spread culture around. Cover with a glass coverslip – be sure to introduce an air bubble.

2. Observe first with the low power objective, focusing on an air bubble.

3. Observe next with the 40X objective, again focusing on the edge of an air bubble. Look for small translucent objects moving in the liquid. Note the shape of the organisms, their clustering, and whether they appear to move independently or just drift along in the currents or vibrate (Brownian motion). Record your observations in the data table.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Shape</th>
<th>Arrangement</th>
<th>Gram stain (+, -, variable)</th>
<th>motility</th>
<th>photo #</th>
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