The following is adapted from "Exploring New Cultures", the Lab Manual for
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LEEUWENHOEK'S "BEASTIES"

Protocols for use of the Light Microscope and Wet Mount Preparation.

Leeuwenhoek squints into his handmade microscope "Come here! Hurry! There are little animals in this rain water...They swim! They play around! They are a thousand times smaller than any creatures we can see with our eyes alone!" (DeKrief, 1954)

INTRODUCTION

Microscopy

Leeuwenhoek, in the late 1600's, was the first to record observations of microorganisms. He announced to world the presence of life in a previously unseen world. His tool was a simple microscope. In the General Microbiology lab we will use an improved version of the microscope: the **brightfield, compound, binocular, light microscope**.

Bright field:	Field of view is illuminated by incident light and appears "bright".
Compound:	Microscope contains more than one lens (ocular and objective lenses).
Binocular:	There are two ocular lenses, one for each eye.
Light:	Visible light is the source of illumination.

The bright field scope is a standard in the microbiology lab and works best when using stained specimens. Unstained specimens have little contrast with their surrounding and are better visualized with modified versions of the light microscope such as the dark field microscope and the phase contrast microscope.

Characteristics of light microscopes:

Bright Field microscope:
Incident light passes from the source through the condenser lens, to the specimen, and on to the objective and ocular lenses. This scope is most commonly used for visualization of stained specimens. Live unstained specimens have little contrast with the background and are better viewed with one of the modified light microscopes.

Dark-field microscope: Light passes through a condenser modified with a *dark field stop*. The stop blocks *incident* light from proceeding to the condenser and results in a dark field. However, if the light hits a specimen, the light will be reflected to the objective lens and passed on to the ocular lens. This gives an image where the specimen is bright in a dark background. Dark Field microscopy is especially useful in viewing live, unstained specimens that are motile, or have an unusual shape.

Phase Contrast microscope: Light passes through a condenser modified with an annular diaphragm. By passing through this diaphragm, light is modified to a hollow cone. This incident light is thus situated to enter the modified condenser of the phase scope. By passing through the phase ring of the condenser, the incident light is advanced 1/4 wavelength. Unlike incident light, light that strikes the specimen is reflected and as a result is will pass through another area of the modified objective. As such the light that

hits the specimen is delayed 1/4 wavelength. The end result is an alteration in the phase of the light which increases contrast between specimen and background. Due to the increased contrast between specimen and background, live specimens are clearly viewed with phase contrast microscopy.

Microorganisms

Microorganisms or Microbes are classified into three domains:

Eukaryotic microbes are found in the domain Eukarya:

PROTOZOA are unicellular, non-photosynthesizing eukaryotic microbes. They are diverse in morphology and lack a rigid cell wall. Examples include amoebae and paramecia.

FUNGI are unicellular or multicellular eucaryotes lacking chlorophyll and usually bearing spores. An example of a filamentous fungi is the mold *Aspergillus niger*. An example of a unicellular, non-filamentous fungi is the yeast *Saccharomyces cerevisiae*. Budding is the mechanism of reproduction for *Saccharomyces*.

ALGAE are unicellular or multicellular eucaryotes that perform photosynthesis and lack tissue differentiation. An example is the unicellular *Chlorella*.

Prokaryotic microbes are found in two domains: Bacteria and Archae.

The domain **BACTERIA** contains procaryotic organisms that contain cell walls with peptidoglycan. They are classified into genera based upon a huge diversity of characteristics many of which we will observe in lab. The majority of prokaryotes that we encounter in our day to day environments are Bacteria. They are found in our soil, on our skin and in our yogurt. One example is *Bacillus cereus*, a rod shaped soil organism.

The domain **ARCHEAE** are procaryotic microbes that do not contain peptidoglycan in their cell walls. In addition, Archeae can be differentiated from Bacteria via analysis of ribosomal DNA sequences. Archeae are found in extreme environments such as acid springs or salt lakes. Microbes in this class are extremely diverse. Each has developed characteristics that allow survival in particular extreme environments. We will not work with Archeae in this lab.

Protocol: USE OF THE BRIGHT-FIELD MICROSCOPE

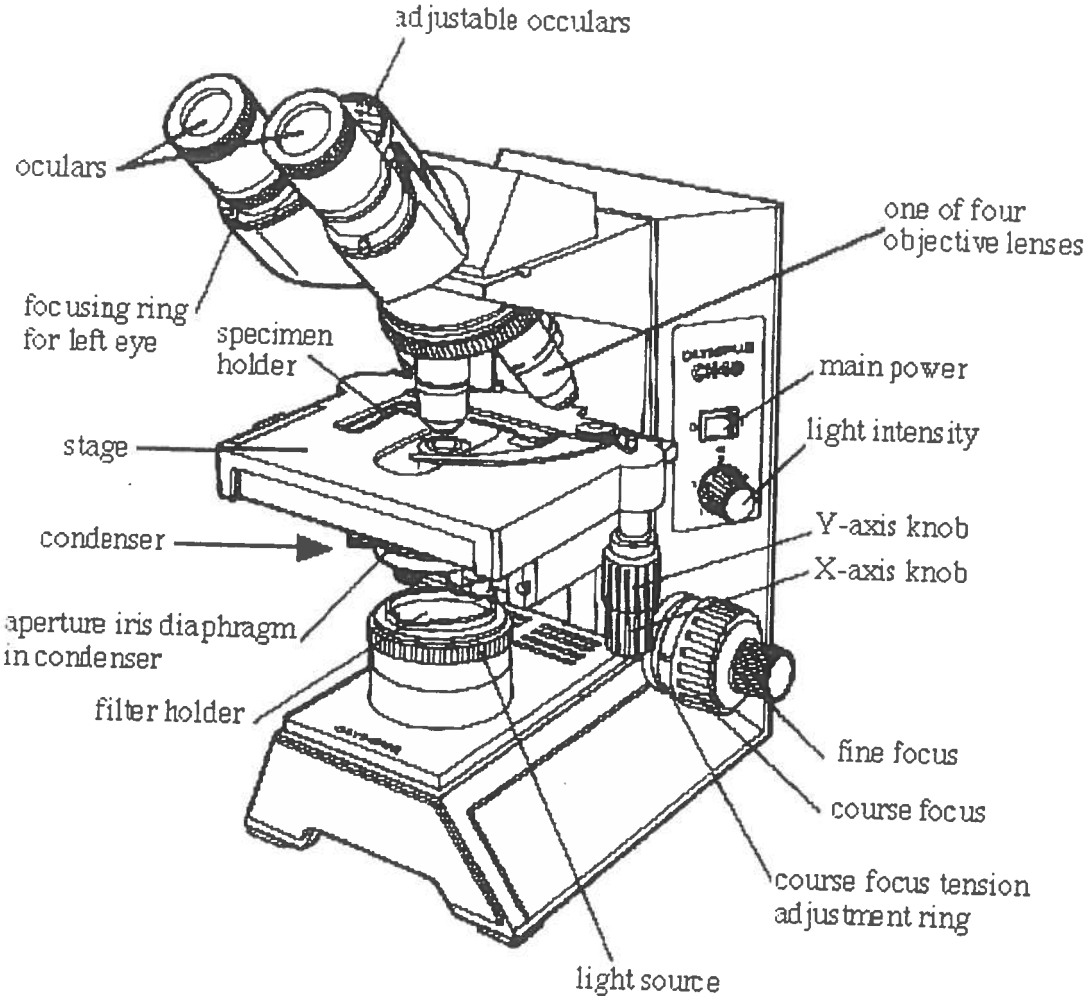
PART A: Locate the following components of the bright-field microscope

- **Arm:** supports the body tube and is the part to grasp when carrying the microscope.
- **Body tube:** connects the ocular lens with the nosepiece housing the objective lenses.
- **Nosepiece:** rotating part to which the three objective lenses are attached. When rotating the objectives, make sure they click into position or no light will pass through.
- **Objectives:** lenses that magnify the images by the power shown on the side of the objective lens: low-power (4X, 10X), high-dry (40X), and oil-immersion (100X).
- **Ocular lens:** lens that magnifies the real image another ten times.
- **Stage:** supports the microscope slide, which is held on to the stage by a slide holder. The stage contains an opening that allows incident light shine through to the specimen.
- **Condenser:** a lens located directly below the microscope stage that concentrates the light before it passes through the specimen. There is a knob that can raise or lower the condenser.
- **Iris diaphragm (Condenser Aperture):** an opening on the bottom of the condenser that regulates the amount of light entering the condenser. A lever moves back and forth to regulate the size of the opening.
- **Coarse adjustment:** a rotating knob that moves the body tube up or down to bring the specimen into initial focus. This knob is used only with the 4X and 10X objectives. With the high-dry (40X) and oil immersion (100X) objectives, there is a danger of driving the objectives through the slide.
- **Fine adjustment:** a smaller rotating knob that brings the specimen into final focus by moving the body tube more slowly. As the objective lenses are parfocal, only the fine adjustment knob needs to be used after you have focused with the low-power objective. (**Parfocal - lenses** are arranged such that after focusing with one objective; the image will remain in focus when moving to another objective.)

PART B: Theory of Magnification and Resolution

Magnification:

Magnification is a property of the lenses of the microscope: the greater the number of lenses, the higher the magnification. The total magnification of a compound microscope is the product of the magnification values for all lenses. Theoretically there is no limit to the magnification value as long as the lens number increases. In practice, however, magnification will eventually be limited by the **resolution (resolving power)** of the microscope. For a clear image, when magnification is increased, the resolving power must also increase.



Resolution (Resolving Power)

The resolving power of a microscope is the ability to distinguish between two objects that are close together. Resolving Power is a function of the **wavelength** of the light source and the **numerical aperture** of the lens. With great resolving power, two points divided by a very small distance will be viewed as separate. The distance between two points, which are seen as distinct, is the **limit of resolution.** When the limit of resolution is small the resolving power of the microscope is great.

Limit of Resolution = (wavelength X 0.61*) /(numerical aperture)

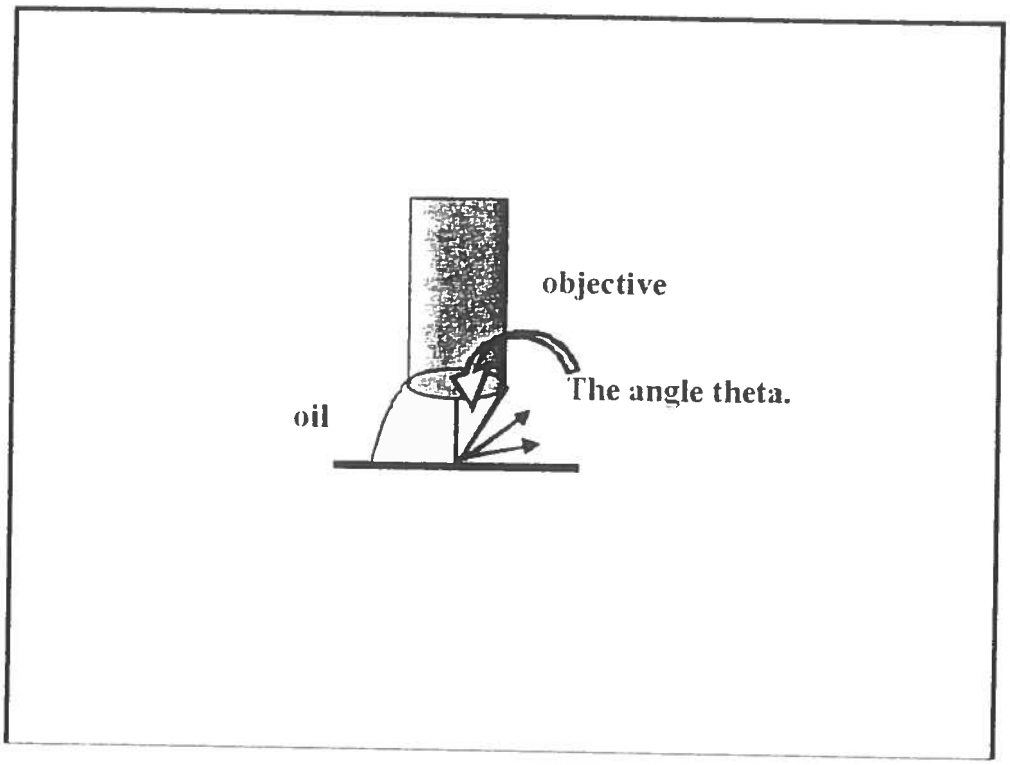
According to this formula, the limit of resolution is least when using shorter wavelengths of illumination. Visible light has a wavelength range of 400-700 nanometers (nm). When using a blue filter to select the shortest wavelength of visible light, the smallest objects that can be resolved by the light microscope are in the range of 0.2 micrometers (μm).

The numerical aperture (NA) is the light capturing ability of the lens. The NA is a function of the sin of the angle theta (see diagram). By examining the numerical aperture equation, it is apparent that the refractive index is the limiting factor in achieving numerical apertures greater than 1.0. Therefore, in order to obtain higher working numerical apertures, the refractive index of the medium between the objective lens and the specimen must be increased. Microscope objectives are now available that allow imaging in alternative media such as immersion oil (refractive index -1.4 vs refractive index of air – 1.0)

***Note**: In the formula above, 0.61 is a constant for the model of microscope used in this class. The diffraction waves around two points may overlap to some extent and the points will still be seen as distinct. This constant accounts for this overlap.

Oil is required for high NA lenses. On your student scope the 100X objective requires oil for proper operation. 4X, 10X and 40X lenses do not require oil. (NOTE: Use of oil immersion on "dry" objectives is improper procedure and will give a blurry image).

Figure 1. Use of oil immersion with high NA lenses. For proper operation of the 100X lens, oil must fill the space between the objective and the specimen.



To view molecules and subcellular structures an electron microscope is used. By using an electron beam (with wavelengths between 0.01 nm and 0.001nm) electron microscopes can resolve objects in the nanometer range.

PART C: Use of the Brightfield microscope

[Clean lenses first using Isopropyl Alcohol and lens paper – to remove bacteria transferred from previous user and oil left on lenses from previous use.]

1. Locate the various parts of the microscope. Place body tube in highest position.
2. Plug in the light source and switch on. Increase light to bright setting.
3. Place the slide on the stage, specimen side up. Secure with specimen clip. Use adjustment knobs under the stage to move the specimen into position over the incident light. Adjust spacing between eye pieces (**interpupillary distance**). Adjust focus for difference in sight between right and left eye (**diopter adjustment**).
4. Looking at your microscope from the side, place the 4X objective into place. Lower the body tube with the coarse adjustment so that the low power objective is just touching the slide.
5. Look through the eyepiece and slowly raise the objective with the coarse adjustment until the specimen is in focus.
6. Use the 4X and 10X objectives to scan the slide and view large microbes. These objectives are also used to adjust focus. Coarse adjustments may be made at this level. Once focus is achieved with the 4X and 10X objectives, very little modification will be needed to see the image with higher power objectives. Adjust condenser aperature to decrease light and increase contrast. After scanning the slide, center a specimen of interest. Open condenser aperture to appropriate setting as indicated on the adjustment knob. View. To view at greater magnification, rotate the nosepiece until the high-dry objective (40X) clicks into place.
7. With increasing magnification, the field of view decreases. Specimens on the edge of the field with the 10X objective will be out of the field of view for the 40X objective. In addition, the working distance between the objective and specimen decreases when high power objectives are used. There is very little space between the objective and the specimen, Do NOT use coarse adjustment focus knob. Minimal focusing adjustments may be achieved with the fine focus knob. Use the 40X objective to find tiny microbes such as bacteria. Observe motility and arrangement of cells. Center an interesting specimen and prepare to examine closely with the oil immersion lens.
8. For use of the 100X oil immersion lens: Rotate the nosepiece so that the *space between* the 40X and 100X lenses is opposite the specimen. Add one full drop of oil to cover the area of the slide over the light and fill the space between the objective lens and the slide. (DO NOT ADJUST STAGE OR FOCUS AT THIS TIME).
9. Rotate the nosepiece so that the oil-immersion objective (100X) clicks into position and is immersed in the oil.
10. Check focus. Make minimal focusing adjustments with the fine adjustment knob. Be careful with large adjustments. There is a very small working distance and it will be very easy to loose the image clarity. If the image is lost, it is best to return to the low power, refocus and follow the steps from low power, to 40X, to 100X objectives.

Points to consider when using the light microscope:

Contrast produced in the specimen by the absorption of light, brightness, reflectance, birefringence, light scattering, diffraction, fluorescence, or color variations has been the classical means of imaging specimens in brightfield microscopy.

Contrast is not an inherent property of the specimen, but is dependent upon interaction of the specimen with light and the efficiency of the optical system coupled to its ability to reliably record this image information with the detector. Control of image contrast in a microscope optical system is dependent upon several factors, primarily the setting of aperture diaphragms, degree of aberration in the optical system, and the type of specimen. There are several sites in the microscope that allow adjustment of contrast: the field aperture, condenser aperture, as well as specimen staining.

It is a common practice to reduce the condenser aperture diaphragm below the recommended size or to lower the substage condenser to increase specimen contrast. This may be helpful as you are searching for specimens on your slide. However while these maneuvers increase contrast, they also seriously reduce resolution and sharpness and may obscure coloring of a specimen. Once you have found a specimen to observe, raise the level of the condenser to the highest level and open the condenser diaphragm to the appropriate degree (see label on diaphragm related to objective in use).

ALWAYS remember to clean the scope when you are finished. Use lens paper to wipe lenses and remove oil. Wrap cord around microscope base, place the 4X objective in place and return the scope to the cabinet.

Protocol: WET MOUNT PREPARATION AND VIEWING USING THE LIGHT MICROSCOPE

MATERIALS

Microscope slides and cover slips; lens paper; sterile toothpicks; *Bacillus* species - rod shaped bacterium; *Saccharomyces cerevisiae* - eucaryote, yeast, reproduces by budding; *Aspergillus niger* - eucaryote, mold, aerial hyphae; *Chlorella* sp. - cukaryote, algae; photosynthetic Hay Infusion (mixed culture)

SPECIMEN PREPARATION -WET MOUNT RECORDING MICROSCOPIC OBSERVATIONS

1. Use a water bottle or inoculating loop to place a small drop of water on the center of a glass slide.
2. Add sample to the drop of water. Use these guidelines to ensure aseptic technique:
    a. **Sterilize dry inoculating loop**: place the loop in the hottest area of the bunsen burner flame (tip of the blue cone). Allow the loop to become red hot. Sterilization is complete. Remove loop from flame. Hold in air to cool.
    b. **Transfer culture from tube:** Remove cap from culture tube. Flame opening of tube. Insert the sterile loop into tube and remove a small amount of culture. Re-flame the opening of the tube and replace cap.
    c. **Transfer culture from loop**: Place loop with sample into the drop of water on the slide. Mix culture into water using loop.
    d. **Sterilize contaminated loop**: insert the wet contaminated loop into the cool area of the bunsen burner flame (cool area is within the blue cone). Allow the wet sample to bake. Now move the loop to the hottest area of the flame (tip of blue cone). Allow the loop to become red hot. Sterilization is complete. Remove loop from flame. Hold in air to cool, replace in receptacle.
3. Carefully place a cover slip over the inoculated drop of water. To do this without creating bubbles in the preparation, first place the cover slip in the drop of water at an angle. Once a meniscus forms across the slip, allow the cover slip to drop into the water.
4. To record microscopic observations, draw a circle to indicate the **field of view**. Label the circle with the total magnification. Do your best to draw observations to scale. It is not necessary to draw everything within the field, Draw only what is pertinent and interesting. Label your drawing to indicate shapes of cells, color, motility, etc.

Staining and Streaking

Protocols for Simple stain, Gram Stain, Streak Plate Technique and Culture Maintenance

INTRODUCTION: STAINING

Live specimens are difficult to see with the bright field microscope. The contrast between a cell, which is *primarily* water, and the background, which *is* water, is poor. Staining is used to increase contrast and can be employed to provide information about the chemistry of a specimen.

Stains, or dyes, are salts in which one of the ions is colored. In a basic stain, the color is in the positively charged ion. In an acidic stain the color is in the negatively charged ion. Bacterial surfaces have a slight negative charge. Thus, there is an affinity between a positively charged color ion and the negatively charged bacterial cell. In the **Direct or Positive Staining Procedure** a cell takes up a positively charged dye and becomes stained. Methylene blue, crystal violet, and safranin, are all basic dyes. In the **Indirect or Negative Staining Procedure,** a cell is immersed in a negatively charged dye. As the cell will repel the dye, the cell appears clear in a background of color. Nigrosine is an example of an acidic stain.

Staining procedures that use *one* dye to increase contrast between specimen and background are **simple** staining procedures. **Complex** staining procedures employ a *series* of stains and chemical reagents to increase contrast and reveal information about the specimen. Any staining procedure that allows differentiation of one type of bacterium from another is a **differential** staining procedure.

For all staining procedures, it is first required that cells be **fixed** to the slide. In light microscopy fixing is completed through heating. In electron microscopy, fixation is accomplished using chemical fixatives. All fixatives kill cells. But their function in microscopy is to preserve bacterial structures while enhancing adherence of cells to the slide.

The **Gram stain**, the most widely used staining procedure in bacteriology, is a complex and differential staining procedure. Through a series of staining and decolorization steps, bacteria are differentiated according to cell wall composition. **Gram-positive** bacteria have cell walls that contain thick layers of peptidoglycan (90% of cell wall). These stain purple. **Gram-negative** bacteria have walls with thin layers of peptidoglycan (10% of wall), and high lipid content. These stain pink. This stain is not used for Archaea or Eukaryotes as both lack "peptidoglycan".

In the Gram stain, after heat fixing, cells are stained with the first or **primary** stain, crystal violet. Both Gram-negative and Gram-positive cells bind this initial positive stain, which forms large complexes inside the cell wall. After washing, a **mordant**, Gram's iodine, is added. Mordants increase the binding affinity of stains. Iodine binds with the crystal violet to form large CV-I, complexes within the cell wall. A **decolorizing agent**, alcohol, is added next. This is the crucial, differentiating step. The dehydrating effect of the alcohol causes the peptidoglycan-rich cell wall of the Gram positive cells to shrink, holding the crystal violet-iodine complexes in the cells. The alcohol dissolves the lipid in the Gram-negative cell walls, leaving the walls porous. Crystal violet-iodine complexes leak out of the Gram-negative cells leaving them without color. At this point the Gram positive cells are purple and the Gram negative cells are clear. A **counterstain**, safranin, is added to allow visualization of the Gram negative cells.

MATERIALS:

Control Cultures: *Staphylococcus epidermidis* – gram-positive cocci
Escherichia coli – gram-negative rod

slides

cover slips

wax pencils

Positive stains: Crystal violet, Safranin

Gram stain reagents: Crystal violet, Gram's iodine, 95% alcohol, Safranin

Protocol: Preparation of sample for staining/ heat fixing.

1) Label slide: use permanent marker to write name of sample on one end of the slide.
2) Add sample to slide:

-For cultures from a plate or a slant:

TA's will demonstrate how to aseptically open and close the plate or slant culture tube.

a. Use a wax pencil to draw lines dividing a slide into thirds. Add one drop of water to each section of the slide. The middle section is for the sample and the other two sections are for a positive and negative control.

b. Using a sterile loop, transfer a small amount of bacterial culture to each water drop and mix. Flame the loop and replace in receptacle.

→Take care to transfer a small amount of culture! Too much culture will interfere with staining procedures. Be sure that the culture is well mixed in the water - there should be no large particles in the preparation.

-For broth cultures:

TA's will demonstrate how to aseptically open and close the broth culture tube.

a. Use a wax pencil to draw lines dividing a slide into thirds. The middle section is for the sample and the other two sections are for a positive and negative control.

b. Using a sterile loop remove one loopful of culture from the tube. Transfer culture to the center of each section of the slide. Flame loop and replace in receptacle.

3) Allow the sample to AIR dry completely.
4) Heat Fix: Hold slide carefully. Pass the slide through the upper-most portion of the Bunsen burner flame. Repeat 2 additional times.

Protocol: Simple Stain

1) Place slide with fixed sample in straining tray, flood slide with a positive stain such as crystal violet. Allow stain to remain on culture for one minute. Gently rinse slide with water, drain.
2) Place slide into the interior of a blotting paper tablet. Close tablet. Apply pressure to blot. Do not rub. (The slide should be completely dry before microscopic examination.)
3) Use Brightfield microscope to examine specimen. Begin with low magnification.
4) Record observations of cells as they appear at 1000X magnification. Observe an area of the slide where individual cells are seen clearly. If no such field can be found on your slide, there are too many cells in your sample. Repeat the sample preparation with fewer cells.

Protocol: Gram Stain (Modified from: ASM Microbe Library Protocol)

Materials:

Slides
Crystal Violet
Gram's Iodine
Ethanol (95%)
Safranin
Gram Stain Controls (a gram-positive organism and a gram-negative organism)

Background:

In aqueous solutions crystal violet dissociates into CV^+ and Cl^- ions that penetrate through the wall and membrane of both gram-positive and gram-negative cells. CV^+ interacts with negatively charged components of bacterial cells, staining the cells purple. When added, iodine (I^- or I_3^-) interacts with CV^+ to form large CVI complexes within the cytoplasm and outer layers of the cell. The decolorizing agent, (ethanol), interacts with the lipids of the membranes of both gram-positive and gram-negative Bacteria. The outer membrane of the gram-negative cell is lost from the cell, leaving the peptidoglycan layer exposed. With ethanol treatment, gram-negative cell walls become leaky and allow the large CV-I complexes to be washed from the cell. The highly cross-linked and multi-layered peptidoglycan of gram-positive cells is dehydrated by the addition of ethanol. The multi-layered nature of the peptidoglycan along with the dehydration from the ethanol treatment traps the large CV-I complexes within the gram-positive cell. After decolorization, the gram-positive cell remains purple in color, whereas the gram-negative cell loses the purple color and is only revealed when the counterstain, the positively charged dye safranin, is added. At this point the Gram negative cell will be pink/red.

Procedure:

1) Prepare slide for staining: Use a wax pencil to divide the slides in thirds. Add samples (water drop and immersed culture from plate OR drop from broth culture) to right and left thirds of the slide for control cultures (gram positive and gram negative controls) and center area for test organism. Air dry, and heat fix.
2) Place slide in staining tray, flood with crystal violet. Stain for 30 seconds.
3) Gently rinse with water and drain.
4) Flood the slide with Gram's iodine. Let stand for 10 seconds to one minute.
5) Rinse with water and drain.
6) Flood slide with alcohol for 30 seconds or until stain no longer washes from the slide when held at an angle.
7) Quickly rinse slide with water.
8) Flood slide with safranin. Allow stain to remain for 30 seconds.
9) Rinse with water and drain.
10) Blot slide within a pad of blotting paper. Do not rub. The slide should be completely dry before microscopic examination.
11) Use 1000X magnification to observe individual bacterial cells.
12) Check the Gram Stain controls. Determine that Gram positive cells are purple, and that Gram negative cells are pink/red . If you have achieved the expected result with the Gram stain controls, observe your test culture. If the Gram stain controls do not stain as expected, repeat the procedure on a freshly prepared slide.

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