OCULAR and STAGE MICROMETERS

Size is one of the most important physical features employed in the identification and characterization of an organism. The exact size of a microorganism can only be determined by utilizing a calibrated **ocular micrometer**. An ocular micrometer (Fig. 2-1) is a glass disc on which a series of uniformly spaced lines has been inscribed. The ocular micrometer is placed in one of the eyepieces of the microscope; however, the distance between the etched lines depends upon the objective lens used to view the specimen. In order to determine the precise distance between the lines of an ocular micrometer, it must be calibrated with a **stage micrometer** (Fig. 2-2). The inscribed lines on a stage micrometer are exactly 0.01 mm (or 10 μm) apart.

In order to calibrate the ocular micrometer for a particular objective lens, the ocular and stage micrometers are superimposed, and the number of ocular graduations per stage micrometer graduation is determined. Figure 2-3 indicates that six ocular micrometer graduations fit between two stage micrometer graduations; therefore, one space of the ocular micrometer is equal to 10 μm/6 or 1.66 μm. In order to determine the dimensions of an organism, the number of graduations occupied by the organism (Fig. 2-4) is counted and multiplied by the distance between graduations. For example, if an organism occupied the space of seven graduations, this particular dimension would be 7 x 1.66 or 11.6 μm.

Very seldom will an exact number of ocular micrometer lines fit between two stage micrometer lines. If that is the case, the number of stage micrometer lines is divided by the total number of ocular micrometer lines in order to determine one ocular graduation. **EXAMPLE**: 25 graduations on the ocular micrometer precisely match 4 graduations on the stage micrometer. Remembering that the graduations of the stage micrometer are 10 μm apart, one ocular graduation = 40/25 = 1.6 μm.

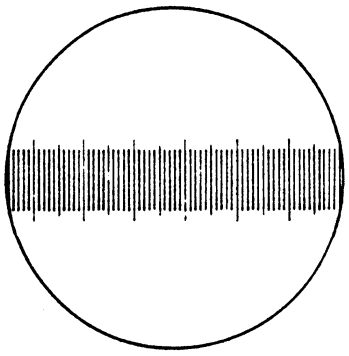


Figure 2-1. Ocular micrometer.

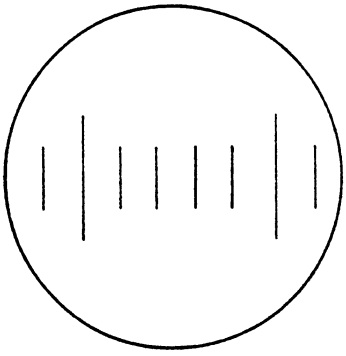


Figure 2-2. Stage micrometer.

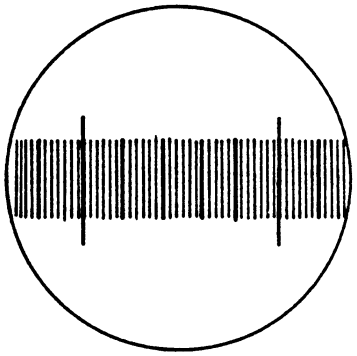


Figure 2-3. Ocular micrometer superimposed on stage micrometer.

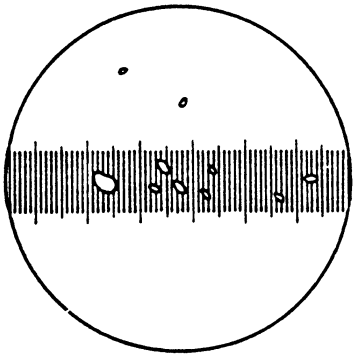


Figure 2-4. Use of the ocular micrometer to measure organisms.

Material:

1. Ocular micrometer
2. Stage micrometer
3. Prepared slides of bacteria

Procedure:

1. Obtain a stage micrometer from the instructor. Place the stage micrometer on the stage of the microscope.

2. Rotate one of the microscope eyepieces until the lines of the ocular micrometer are parallel with those of the stage micrometer.

3. Match the lines on the left edges of the two micrometers by moving the stage micrometer so that the graduations of the ocular micrometer are superimposed over those of the stage micrometer.

4. Determine the number of ocular micrometer spaces that fall within a given number of stage micrometer spaces.

5. Calculate the distance between each ocular graduation by using the following formula:

$$1 \text{ ocular micrometer space } (\mu m) = \frac{x \text{ spaces on the stage micrometer}}{y \text{ spaces on the ocular micrometer}}$$

6. Repeat the procedure for the 10X and 40X objectives and record results. **Estimate** the calibration of the 100X oil-immersion objective lens by dividing the value for the 10X objective by 10.

Results:

	One ocular micrometer graduation equal to
4X objective	
10X objective	
40X objective	
100X objective	

SIMPLE STAINING and BACTERIAL MORPHOLOGY

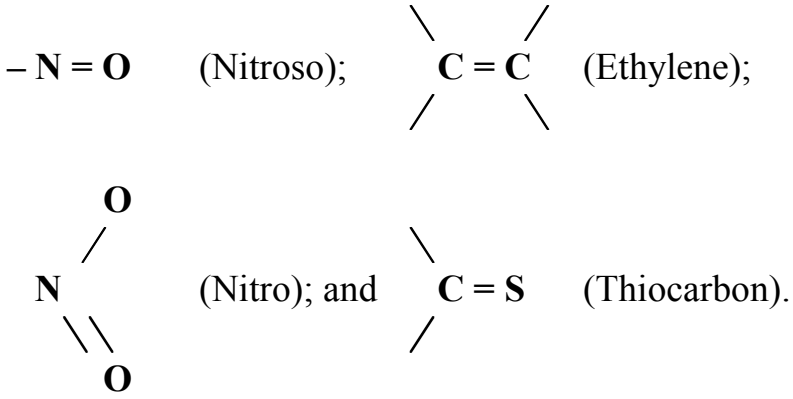
Since bacteria are typically 0.2 to 2.0 μm on longest dimension, they are difficult to see with the light microscope. Bacterial cells in the "living state" were first observed by Leeuwenhoek in drops of fluid. Wet mounts were commonly used by Pasteur, Lister and many of the early workers in the field of microbiology. The method was adequate and even desirable for certain observations, but the constant motion of cells, due either to natural motility or Brownian movement, made accurate morphological studies very difficult. Robert Koch, during his classical research on anthrax, prepared thin films of bacteria on glass and allowed the smears to dry. Although the anthrax bacilli were motionless and unchanged in general shape, the more detailed structure of the cells was still obscure, due to their colorless and transparent nature. The simplest way to increase contrast with the surrounding media was to use dyes which are taken up by the bacterial cells. In 1875, Weigert, a German contemporary of Koch, found that the dye, methyl violet, would color or "stain" bacterial cells in some tissue preparations. This use of dyes to render bacterial cells easily visible under the microscope was adopted by Koch and his many students, and soon became one of the most widely used fundamental techniques of microbiology.

Prior to staining with dyes, cells must be "fixed." Fixation is a process of killing, immobilizing and preserving the bacterial cell. For bacteria, heat fixation is most common; however, chemicals such as formaldehyde, glutaraldehyde, acids and alcohols can be used.

Dyes used to stain bacterial cells are organic compounds which have affinity for specific cellular components. Many dyes are positively charged (cationic) and combine strongly with negatively charged cellular materials such as nucleic acids and acidic polysaccharides. Other dyes are anionic (negatively charged) and combine with positively charged constituents such as proteins. A dye is composed of two components:

1. An **auxochrome** group which in itself does not produce color but gives the dye its acidic or basic properties. Examples are NH_2; OH; OCH_3; I; Br; and Cl.

2. A **chromophore** group which imparts color to the dye molecule. Examples are



Simple staining is merely the use of a dye to increase the contrast of cells for microscopy. As an example, a simple stain would be used to detect the presence of bacteria in some natural material such as urine or water. It must be remembered that a simple stain alone is **not** useful as an identification tool. The purpose of this exercise is to illustrate the use of simple stains in the study of bacterial morphology.

Material:

1. Bacterial cultures
2. Crystal violet, safranin

Procedure:

1. Clean microscope slides with soap and water, rinse, and then blot dry.

2. Using an inoculating loop, put a drop of water on the slide.

3. Flame, i.e. sterilize, an inoculating loop and using **aseptic technique**, remove a small amount of bacteria from some solid medium and mix with the drop of water. The smear should be about the size of a dime and must be fairly dilute. If the smear is too dense, the morphology of individual cells will be impossible to determine.

4. Allow the smear to **air-dry**.

5. Heat-fix the slide by passing it through the flame of a Bunsen burner two or three times, or until the slide is slightly warm when touched to the back of the hand. Heat coagulates the protein of the cells so that the cells stick to the glass surface and are not washed off during the staining and rinsing procedures. Do not overheat the slide, however, as this may cause distortion of cell shape and uneven staining.

6. Allow the slide to cool, then flood with a stain - either safranin for 2 min or crystal violet for 1 min.

7. At the end of the staining time, pour off the stain and wash the slide gently but thoroughly with tap water. Blot dry with a paper towel.

8. Examine the dry smear using low-power, high-power and oil-immersion objectives. Describe the cellular morphology of each bacterial species.

HANGING DROP TECHNIQUE

Most bacterial microscopic preparations kill the organisms. The hanging drop technique allows you to observe the size, arrangement, and shape of **living** cells and to determine motility.

A thin ring of petroleum jelly is applied to the four edges on one side of a cover slip. A drop of pond scum or a drop from a bacterial broth culture is then placed in the center of the cover slip. A concave (depression) microscope slide is carefully placed over the cover slip in such a way that the drop is suspended and is undisturbed. The petroleum jelly causes the cover slip to stick to the slide. The slide preparation may now be inverted and placed under the microscope for examination. Since no stain is used and most cells are transparent, viewing is best done with as little illumination as possible. The petroleum jelly form an air-tight seal that prevents drying of the drop allowing a long period to observe cell size and shape, binary fission, and motility.

If the technique is used to determine motility, the observer must be careful to distinguish between true motility and Brownian motion. Brownian motion is caused by water molecules colliding with the organism and moving it around in an irregular jerky pattern. Organisms will appear to vibrate in place. With true motility, cells will exhibit independent movement in some consistent direction over greater distances.

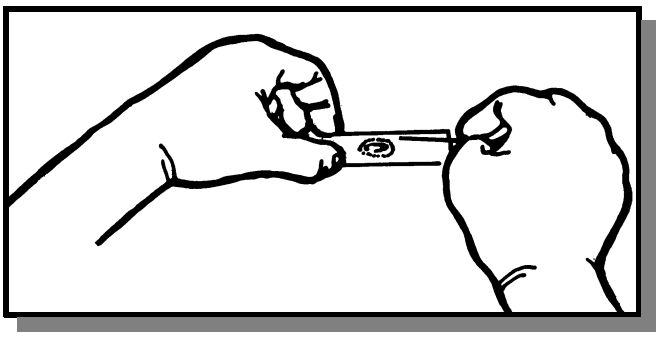
FIRST PERIOD

Material:

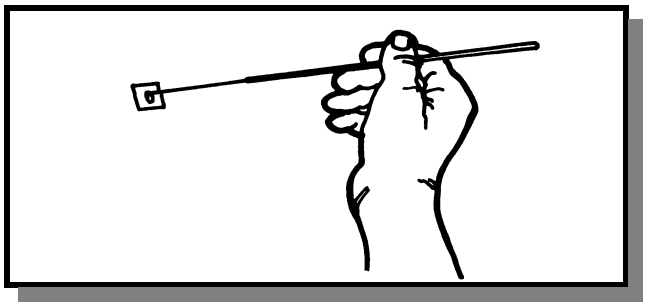
1. Depression slide and cover slip
2. Petroleum jelly
3. Toothpick
4. Pond scum or 24-h bacterial broth culture

Procedure:

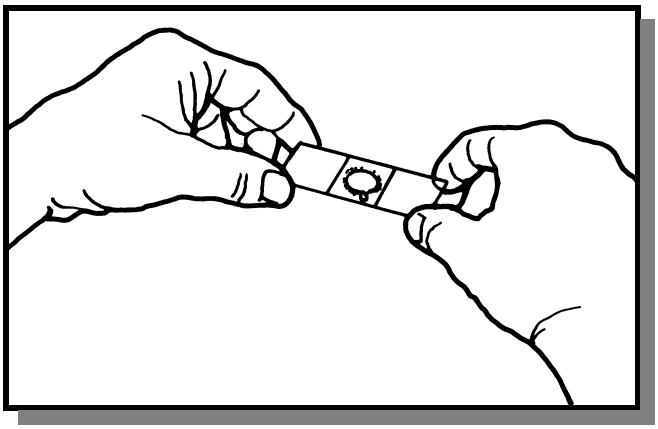
1. *Apply the petroleum jelly*. Use a toothpick to place a thin ring of petroleum jelly around the well of a depression slide. An easy short cut is to only spot the petroleum jelly on the four corners of the cover slip, though drying can then occur.



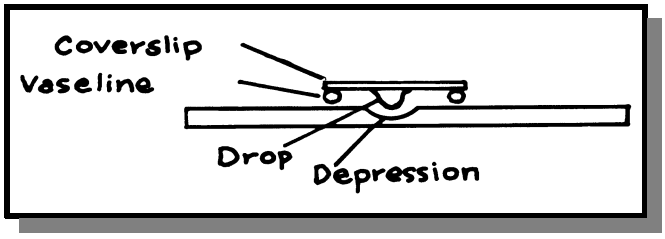
2. *Transfer the Organisms.* Use your loop to place a drop of pond scum on a cover slip. Do not spread out the drop.



3. *Invert the Slide over the cover slip.* Carefully invert the depression slide over the cover slip so the drop is centered in its well. Gently press until the petroleum jelly has created a seal between the slide and the cover slip.



4. *Observe the slide.* Correctly done, the slide should look like this from the side. Place the slide with the cover slip up on the microscope stage and observe under high dry or oil immersion.



DIFFERENTIAL STAINING, Part I

Differential staining is a procedure that takes advantage of differences in the physical and chemical properties of different groups of bacteria. It allows us to differentiate between different kinds of bacterial cells or different parts of a bacterial cell.

I. GRAM STAIN

The most commonly used differential stain is the Gram stain, first described in 1884 by Christian Gram, a Danish physician. The Gram reaction divides bacteria into two groups, those which are Gram-positive and those which are Gram-negative. Those organisms which retain the primary stain (crystal violet) are stained purple and are designated Gram-positive; those which lose the crystal violet and are subsequently stained by a safranin counterstain appear red and are designated Gram-negative.

The conventional Gram-stain technique is described in the **Procedure** part of this handout; however, it is important to recognize early on that two aspects of the procedure are crucial:

1. The crystal violet treatment **must** precede iodine treatment. Iodine acts as a mordant, i.e., it increases the affinity of the cells for the crystal violet. Iodine alone has no bacterial staining capabilities.

2. Decolorization must be short and precise. Too long an exposure to 95% alcohol will decolorize Gram-positive as well as Gram-negative cells.

The Gram stain has been used as a taxonomic tool for many years, aiding in the classification and identification of bacterial cells. However, it is also useful in a broader sense, as there appears to be a close correlation between the Gram reaction and many other morphological and physiological characteristics of bacterial cells. For example, most of the rod-shaped, aerobic, spore-forming bacteria which are isolated from soil show a Gram-positive reaction, whereas most of the rod-shaped (bacillus-shaped) organisms which can ferment lactose and are

common in the intestinal tract of humans demonstrate a Gram-negative reaction. In addition, deadly proteinaceous exotoxins are typically synthesized by Gram-positive pathogens, whereas production of endotoxins seem to be a characteristic of Gram-negative cells.

The cell walls of the two groups are morphologically and chemically quite different. One explanation for the differential staining reaction emphasizes the higher lipid content of the cell walls in Gram-negative bacteria. During the decolorization step, alcohol may extract the lipids, increasing the porosity or permeability of the cell walls. Thus, the crystal violet-iodine complex is easily lost. The Gram-positive bacteria, however, do not have lipid-rich cell walls. Their cell walls become dehydrated during the alcohol treatment, decreasing the porosity so that the crystal violet-iodine complex is retained.

It is important to note that Gram-positive organisms are not always constant in their staining reaction. Older cultures of some Gram-positive bacteria are subject to autolysis. Enzymatic breakdown of the cell wall causes older cells to become Gram-variable (both red and purple cells present) or Gram-negative. The pH of the culture medium will also influence the staining of Gram-positive cells. Cultures for Gram staining should be grown in media low in sugars to avoid the formation of acidic end products during cell growth.

Material:

1. 24-48 hour broth cultures of *Staphylococcus aureus*, *Bacillus megaterium* or *B. subtilis*, and *Escherichia coli*
2. Gram stain reagents –
   a. crystal violet
   b. Gram's (or Lugol's) iodine solution
   c. 95% alcohol
   d. safranin

Procedure:

1. Prepare smears of the organisms listed above and heat-fix smear in the usual way.

2. Allow the slide to cool, then flood with crystal violet. After one minute, pour off the stain and wash the slide gently but thoroughly with tap water.

3. Flood the smear with Gram's (or Lugol's) iodine. After one minute, pour off the iodine solution and rinse with tap water. At this point, try to remove most of the excess water.

4. Decolorize with 95% alcohol. Hold the slide at an angle and "drip" the alcohol over the smear. Observe these drips. As soon as the drips lose the faint touch of blue, rinse the slide with tap water **immediately**. The decolorization step should not have taken more than 15-20 seconds.

5. Counterstain with safranin for 1 or 2 minutes and wash gently with tap water.

6. Blot dry and examine the smear under oil immersion. Record results including sizes of each morphotype in microns.