الجامعة المستنصرية

كلية العلوم

قسم علوم الحياة

المرحلة الثانية

التقنيات المجهرية

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THE MICROSCOPIC TECHNIQUES

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Microscopic techniques

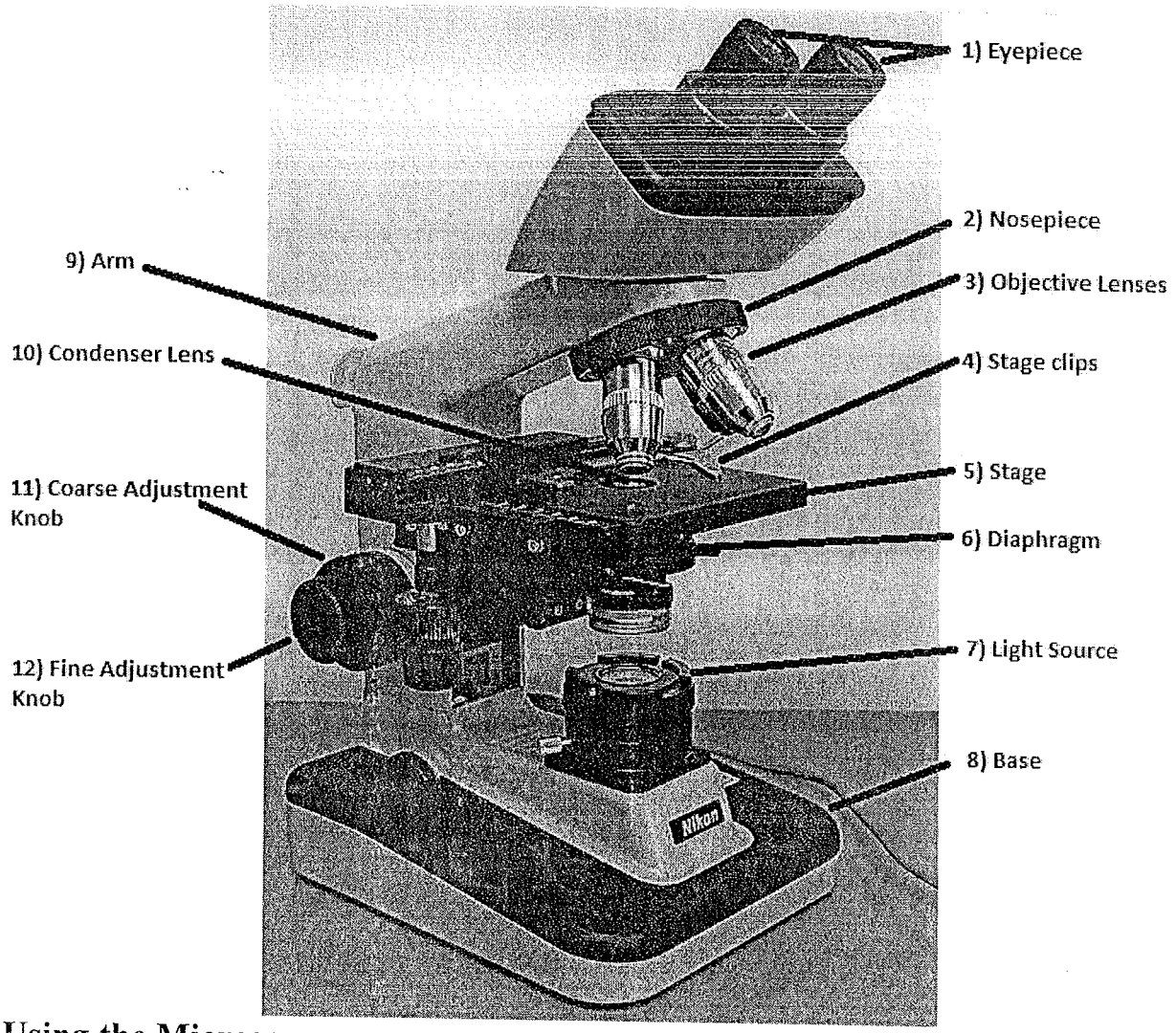
Lab- 1

Compound Light Microscope:

The microscope pictured down is referred to as a compound light microscope. The term *light* refers to the method by which light transmits the image to your eye. *Compound* deals with the microscope having more than one lens. *Microscope* is the combination of two words; "micro" meaning small and "scope" meaning view.

Compound Microscope Parts:

1. **Eyepiece**: contains the ocular lens, which provides a magnification power of 10X to 15X, usually. This is where you look through.
2. **Nosepiece**: holds the objective lenses and can be rotated easily to change magnification.
3. **Objective lenses**: usually, there are three or four objective lenses on a microscope, consisting of 4X, 10X, 40X and 100X magnification powers. In order to obtain the total magnification of an image, you need to multiply the eyepiece lens power by the objective lens power. So, if you couple a 10X eyepiece lens with a 40X objective lens, the total magnification is of 10X 40 = 400 times.
4. **Stage clips**: hold the slide in place.
5. **Stage**: it is a flat platform that supports the slide being analyzed.
6. **Diaphragm**: it controls the intensity and size of the cone light projected on the specimen. As a rule of thumb, the more transparent the specimen, less light is required.
7. **Light source**: it projects light upwards through the diaphragm, slide and lenses.
8. **Base**: supports the microscope.
9. **Condenser lens**: it helps to focus the light onto the sample analyzed. They are particularly helpful when coupled with the highest objective lens.
10. **Arm**: supports the microscope when carried.
11. **Coarse adjustment knob**: when the knob is turned, the stage moves up or down, in order to coarse adjust the focus.
12. **Fine adjustment knob**: used fine adjust the focus.



Using the Microscope

Follow these directions when using the microscope!

1. To carry the microscope grasp the microscopes arm with one hand. Place your other hand under the base.
2. Place the microscope on a table with the arm toward you.
3. Turn the coarse adjustment knob to raise the body tube.
4. Revolve the nosepiece until the low-power objective lens clicks into place.
5. Adjust the diaphragm. While looking through the eyepiece, also adjust the mirror until you see a bright white circle of light.
6. Place a slide on the stage. Center the specimen over the opening on the stage. Use the stage clips to hold the slide in place.
7. Look at the stage from the side. Carefully turn the coarse adjustment knob to lower the body tube until the low power objective almost touches the slide.
8. Looking through the eyepiece, VERY SLOWLY the coarse adjustment knob until the specimen comes into focus.

9. To switch to the high power objective lens, look at the microscope from the side. CAREFULLY revolve the nosepiece until the high-power objective lens clicks into place. Make sure the lens does not hit the slide
9. To switch to the high power objective lens, look at the microscope from the side. CAREFULLY revolve the nosepiece until the high-power objective lens clicks into place. Make sure the lens does not hit the slide.
10. Looking through the eyepiece, turn the fine adjustment knob until the specimen comes into focus.

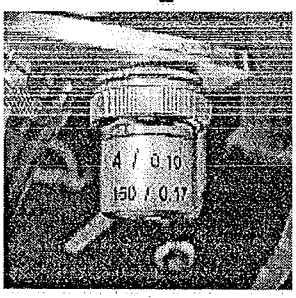
Magnification:

1. When viewing a slide through the microscope make sure that the stage is all the way down and the 4X scanning objective is locked into place.
2. Place the slide that you want to view over the aperture and gently move the stage clips over top of the slide to hold it into place.
3. Beginning with the 4X objective, looking through the eyepiece making sure to keep both eyes open (if you have trouble cover one eye with your hand) slowly move the stage upward using the coarse adjustment knob until the image becomes clear. This is the only time in the process that you will need to use the coarse adjustment knob. The microscopes that you will be using are parfocal, meaning that the image does not need to be radically focused when changing the magnification.
4. To magnify the image to the next level rotate the nosepiece to the 10X objective. While looking through the eyepiece focus the image into view using only the fine adjustment knob, this should only take a slight turn of the fine adjustment knob to complete this task.

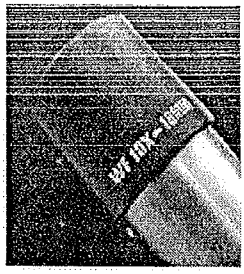
To magnify the image to the next level rotate the nosepiece to the 40X objective. While looking through the eyepiece focus the image into view using only the fine adjustment knob, this should only take a slight turn of the fine adjustment knob to complete this task.

Total Magnification:
To figure the total magnification of an image that you are viewing through the microscope is really quite simple. To get the total magnification take the power of the objective (4X, 10X, 40X) and multiply by the power of the eyepiece, usually 10X.

Total Magnification:

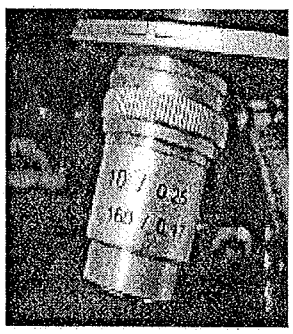


X



= 40 X

4X Scanning Objective 10X Eyepiece



X

= 100 X

10X Objective 10X Eyepiece

X

= 400X

40X Objective 10X Eyepiece

Lab-2
Microscopic Measurements

With an ocular micrometer properly installed in the eyepiece of your microscope, it is a simple matter to measure the size of microorganisms that are seen in the microscopic field. An **ocular micrometer** consists of a circular disk of glass that has graduations engraved on its upper surface. These graduations appear as shown in illustration B, figure 5.4. On some microscopes one has to disassemble the ocular so that the disk can be placed on a shelf in the ocular tube between the two lenses. On most microscopes, however, the ocular micrometer is simply inserted into the bottom of the ocular, as shown in figure 5.1. Before one can use the micrometer it is necessary to calibrate it for each of the objectives by using a stage micrometer.

The principal purpose of this exercise is to show you how to calibrate an ocular micrometer for the various objectives on your microscope. Proceed as follows:

CALIBRATION PROCEDURE

The distance between the lines of an ocular micrometer is an arbitrary value that has meaning only if the ocular micrometer is calibrated for the objective that is being used. A **stage micrometer** (figure 5.2), also known as an *objective micrometer,* has lines scribed on it that are exactly 0.01 mm (10 _m) apart. Illustration C, figure 5.4 reveals the appearance of these graduations. To calibrate the ocular micrometer for a given objective, it is necessary to superimpose the two scales and determine how many of the ocular graduations coincide with one graduation on the scale of the stage micrometer. Illustration A in figure 5.4 shows how the two scales appear when they are properly aligned in the microscopic field. In this case, seven ocular divisions match up with one stage micrometer division of 0.01 mm to give an ocular value of 0.01/7, or 0.00143 mm. Since there are 1000 micrometers in 1 millimeter, these divisions are 1.43 _m apart.

With this information known, the stage micrometer is replaced with a slide of organisms to be measured. Illustration D, figure 5.4, shows how a field of microorganisms might appear with the ocular mi-crometer in the eyepiece. To determine the size of an organism, then, it is a simple matter to count the graduations and multiply this number by the known distance between the graduations. When calibrating the objectives of a icroscope, proceed as follows.

Materials:

ocular micrometer or eyepiece that contains a micrometer disk stage micrometer

1. If eyepieces are available that contain ocular micrometers, replace the eyepiece in your microscope with one of them. If it is necessary to insert an ocular micrometer in your eyepiece, find out from your instructor whether it is to be inserted below the bottom lens or placed between the two lenses within the eyepiece. In either case, great care must be taken to avoid dropping the eyepiece or reassembling the lenses incorrectly. *Only with your instructor's prior approval shall eyepieces be disassembled.* Be sure that the graduations are on the upper surface of the glass disk.
2. Place the stage micrometer on the stage and center it exactly over the light source.
3. With the low-power (10x) objective in position, bring the graduations of the stage micrometer into focus, *using the coarse adjustment knob. Reduce the lighting.*
Note: If the microscope has an automatic stop, do not use it as you normally would for regular microscope slides. The stage micrometer slide is too thick to allow it to function properly.
4. Rotate the eyepiece until the graduations of the ocular micrometer lie parallel to the lines of the stage micrometer.
5. If the **low-power objective** is the objective to be calibrated, proceed to step 8.
6. If the **high-dry objective** is to be calibrated, swing it into position and proceed to step 8.
7. If the **oil immersion lens** is to be calibrated, place a drop of immersion oil on the stage micrometer, swing the oil immersion lens into position, and bring the lines into focus; then, proceed to the next step.
8. Move the stage micrometer laterally until the lines at one end coincide. Then look for another line on the ocular micrometer that coincides *exactly* with one on the stage micrometer.
Occasionally one stage micrometer division will include an even number of ocular divisions, as shown in illustration A. In most instances, however, several stage graduations will be involved.
In this case, divide the number of stage micrometer divisions by the number of ocular divisions that coincide. The figure you get will be that part of a stage micrometer division that is seen in an ocular division. This value must then be multiplied by 0.01 mm to get the amount of each ocular division.
Example: 3 divisions of the stage micrometer line up with 20 divisions of the ocular micro-meter.
Each ocular division = $3/20 \times 0.01$
$= 0.0015$ mm
$= 1.5$ *t*m
9. Replace the stage micrometer with slides of organisms to be measured.

MEASURING ASSIGNMENTS

Organisms such as protozoan, algae, fungi, and bacteria in the next few exercises may need to be measured.

If your instructor requires that measurements be made, you will be referred to this exercise.

Later on you will be working with unknowns. In some cases measurements of the unknown organisms will be pertinent to identification.

If trial measurements are to be made at this time, your instructor will make appropriate assignments.

Important: Remove the ocular micrometer from your microscope at the end of the laboratory period.

Figure 5.1 Ocular micrometer with retaining ring is Inserted into base of eyepiece.

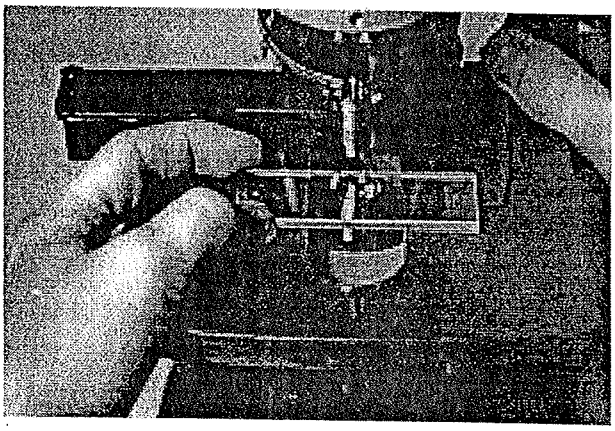


Figure 5.2 Stage micrometer is positioned by centering small glass disk over the light source.

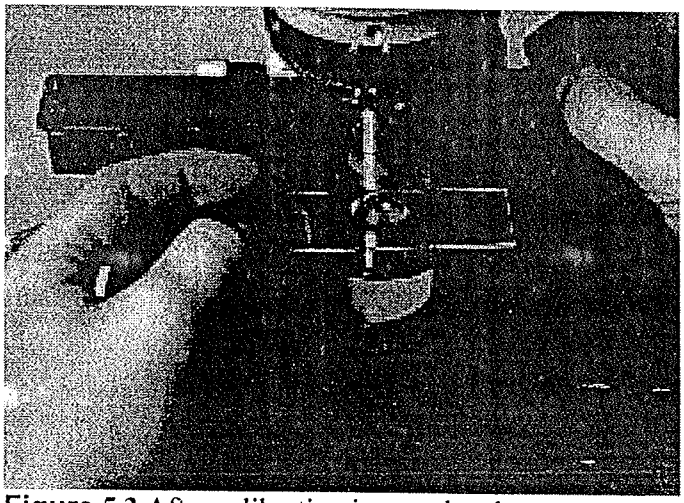


Figure 5.3 After calibration is completed, stage Micrometer is replaced with slide for measurements.

Smear Preparation

EXERCISE 10

The success for most staining procedures depends upon the preparation of a good **smear.** There are several goals in preparing a smear. The first goal is to cause the cells to adhere to the microscope slide so that they are not washed off during subsequent staining and washing procedures. Second, it is important to insure that shrinkage of cells does not occur during staining, otherwise distortion and artifacts can result. A third goal is to prepare thin smears because the thickness of the smear will determine if you can visualize individual cells, their arrangement, or details regarding microstructures associated with cells. Thick smears of cells with large clumps obscure details about individual cells and, furthermore, the smear can entrap stain keeping it from being removed by washing or destaining, leading to erroneous results. The procedure for making a smear is illustrated in figure 10.1.

The first step in preparing a bacteriological smear differs according to the source of the organisms. If the bacteria are growing in a liquid medium (broths, milk, saliva, urine, etc.), one starts by placing two or more loopfuls of the liquid medium directly on the slide.

From solid media such as nutrient agar, blood agar, or some part of the body, one starts by placing one or two loopfuls of water on the slide and then using an inoculating loop to disperse the organisms in the water. Bacteria growing on solid media tend to cling to each other and must be dispersed sufficiently by dilution in water; unless this is done, the smear will be too thick. *The most difficult concept for students to understand about making slides from solid media is that it takes only a very small amount of material to make a good smear.* When your instructor demonstrates this step, pay very careful attention to the amount of material that is placed on the slide.

The organisms to be used for your first slides may be from several different sources. If the plates from Exercise 6 were saved, some slides may be made from them. If they were discarded, the first slides may be made for Exercise 11, which pertains to simple staining. Your instructor will indicate which cultures to use.

FROM LIQUID MEDIA

(Broths, saliva, milk, etc.)

If you are preparing a bacterial smear from liquid media, follow this routine, which is depicted on the left side of figure 10.1.

MATERIALS

- microscope slides
- Bunsen burner
- wire loop
- Sharpie marking pen
- slide holder (clothespin)

1. Wash a slide with soap or Bon Ami and hot water, removing all dirt and grease. Handle the clean slide by its edges.
2. Write the initials of the organism or organisms on the left-hand side of the slide with a marking pen.
3. To provide a target on which to place the organisms, make a $\frac{1}{2}''$ circle on the *bottom* side of the slide, centrally located, with a marking pen. Later on, when you become more skilled, you may wish to omit the use of this "target circle."
4. Shake the culture vigorously and transfer two loopfuls of organisms to the center of the slide over the target circle. Follow the routine for inoculations shown in figure 10.2. *Be sure to flame the loop after it has touched the slide.*

> **CAUTION:** Be sure to cool the loop completely before inserting it into a medium. A loop that is too hot will spatter the medium and move bacteria into the air.

5. Spread the organisms over the area of the target circle.
6. Allow the slide to dry by normal evaporation of the water. Don't apply heat.

7. After the smear has become completely dry, place the slide in a clothespin and pass it several times through the flame of a Bunsen burner. Avoid prolonged heating of the slide as it can shatter from excessive exposure to heat. The underside of the slide should feel warm to the touch.

Note that in this step one has the option of using or not using a clothespin to hold the slide. *Use the option preferred by your instructor.*

FROM SOLID MEDIA

When preparing a bacterial smear from solid media, such as nutrient agar or a part of the body, follow this routine, which is depicted on the right side of figure 10.1.

MATERIALS

- microscope slides
- inoculating needle and loop

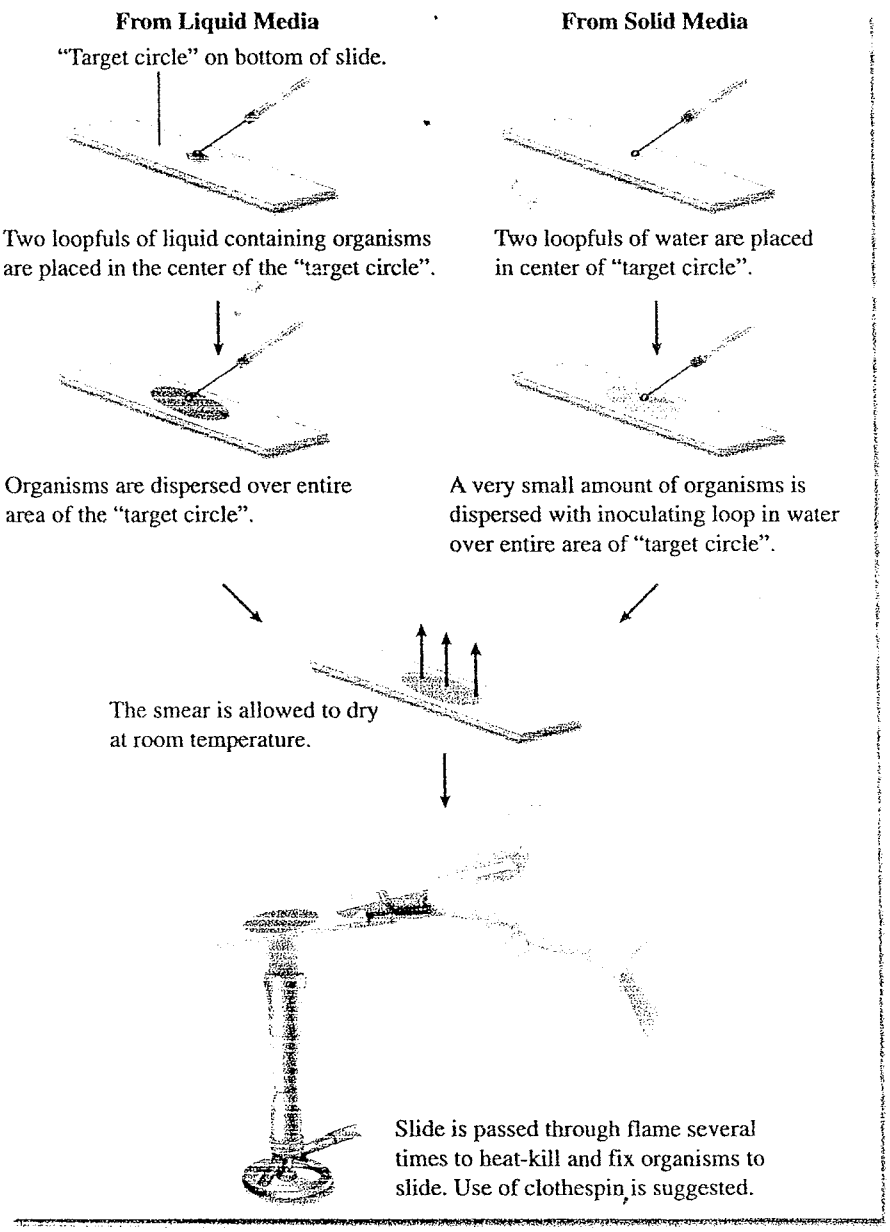


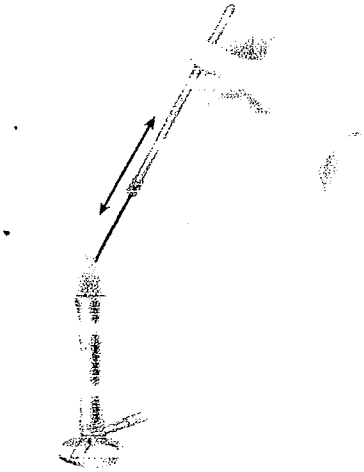
FIGURE 10.1 Procedure for making a bacterial smear

10

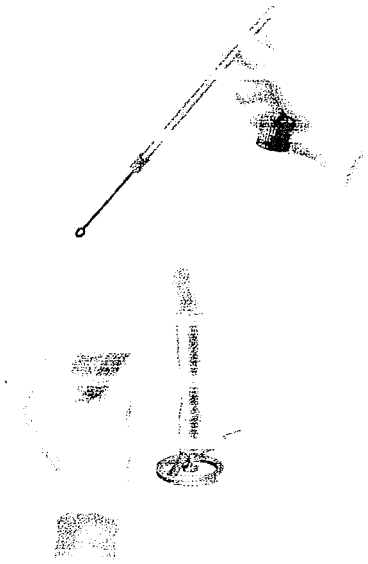
- Sharpie marking pen
- slide holder (clothespin)
- Bunsen burner

1. Wash a slide with soap or Bon Ami and hot water, removing all dirt and grease. Handle the clean slide by its edges.
2. Write the initials of the organism or organisms on the left-hand side of the slide with a marking pen.
3. Mark a "target circle" on the bottom side of the slide with a marking pen. (See comments in step 3 on page 87.)

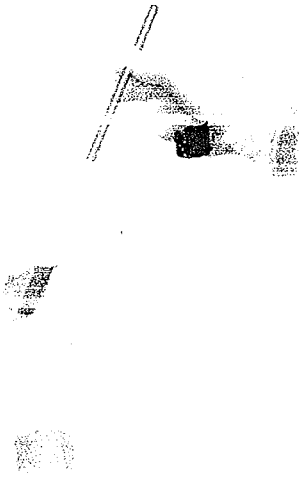
(1) Shake the culture tube from side to side to suspend organisms. Do not moisten cap on tube.



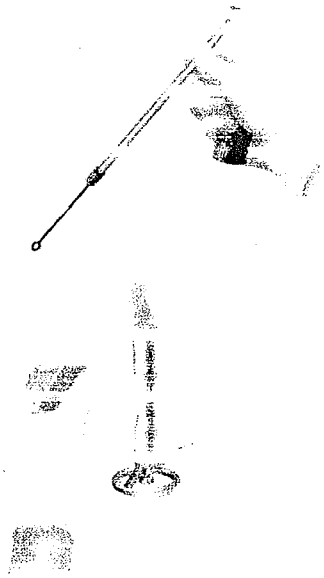
(2) Heat loop and wire to red-hot. Flame the handle slightly also.



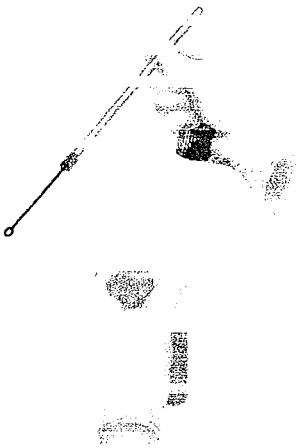
(3) Remove the cap and flame the neck of the tube. Do not place the cap down on the table.



(4) After allowing the loop to cool for at least 5 seconds, remove a loopful of organisms. Avoid touching the side of the tube.



(5) Flame the mouth of the culture tube again.

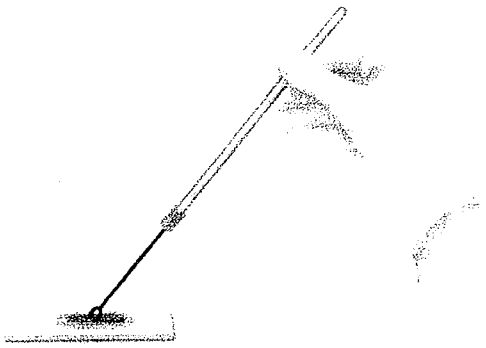


(6) Return the cap to the tube and place the tube in a test-tube rack.

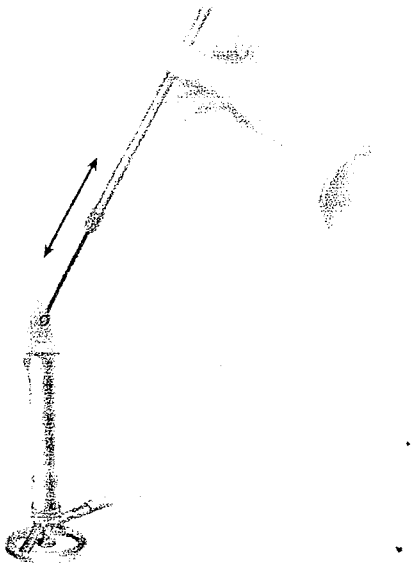
continued

FIGURE 10.2 Aseptic procedure for organism removal

11



(7) Place the loopful of organisms in the center of the target circle on the slide.



(8) Flame the loop again before removing another loopful from the culture or setting the inoculating loop aside.

FIGURE 10.2 *(continued)*

4. Flame an inoculating loop, let it cool, and transfer two loopfuls of water to the center of the target circle.
5. Flame an inoculating needle then let it cool. Pick up *a very small amount of the organisms,* and mix it into the water on the slide. Disperse the mixture over the area of the target circle. Be certain that the organisms have been well emulsified in the liquid. *Be sure to flame the inoculating loop before placing it in its holder.*
6. Allow the slide to dry by normal evaporation of the water. Don't apply heat.
7. After the slide has become completely dry, place it in a clothespin and pass it several times through the flame of a Bunsen burner. Avoid prolonged heating of the slide as it can shatter from excessive exposure to heat. The underside of the slide should feel warm to the touch.

LABORATORY REPORT

Answer the questions on Laboratory Report 10–13 that relate to this exercise.

12

Simple Staining

EXERCISE 11

The use of a single stain to color a bacterial cell is commonly referred to as **simple staining.** Some of the most commonly used dyes for simple staining are methylene blue, basic fuchsin, and crystal violet. All of these dyes work well on bacteria because they have color-bearing ions (*chromophores*) that are positively charged (cationic).

The fact that bacteria are slightly negatively charged produces a pronounced attraction between these cationic chromophores and the organism. Such dyes are classified as **basic dyes.** The basic dye methylene blue (methylene^{+} chloride^{-}) will be used in this exercise. Those dyes that have anionic chromophores are called **acidic dyes.** Eosin (sodium^{+} eosinate^{-}) is such a dye. The anionic chromophore, eosinate^{-}, will not stain bacteria because of the electrostatic repelling forces that are involved.

The staining times for most simple stains are relatively short, usually from 30 seconds to 2 minutes, depending on the affinity of the dye. After a smear has been stained for the required time, it is washed off gently, blotted dry, and examined directly under oil immersion. Such a slide is useful in determining basic morphology and the presence or absence of certain kinds of granules.

An avirulent strain of *Corynebacterium diphtheriae* will be used here for simple staining. In its pathogenic form, this organism is the cause of diphtheria, a very serious disease. One of the steps in identifying this pathogen is to do a simple stain of it to demonstrate the following unique characteristics: pleomorphism, metachromatic granules, and palisade arrangement of cells.

Pleomorphism pertains to irregularity of form: that is, demonstrating several different shapes. While *C. diphtheriae* is basically rod-shaped, it also appears club-shaped, spermlike, or needle-shaped. *Bergey's Manual* uses the terms "pleomorphic" and "irregular" interchangeably.

Metachromatic granules are distinct reddish-purple granules within cells that show up when the organisms are stained with methylene blue. These granules are masses of *volutin,* a polymetaphosphate.

Palisade arrangement pertains to parallel arrangement of rod-shaped cells. This characteristic, also called "picket fence" arrangement, is common to many corynebacteria.

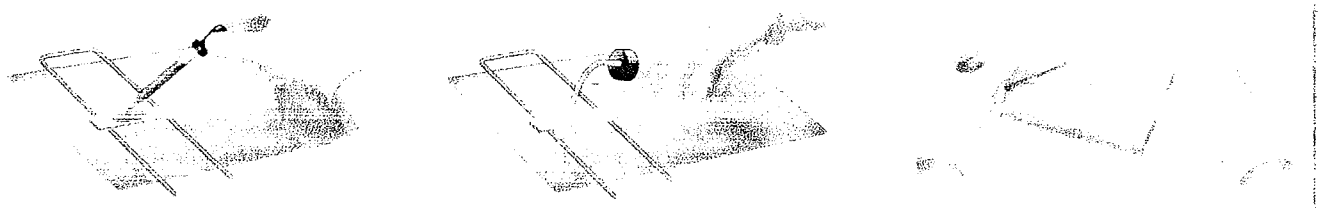
PROCEDURE

Prepare a slide of *C. diphtheriae,* using the procedure outlined in figure 11.1. It will be necessary to refer back to Exercise 10 for the smear preparation procedure.

MATERIALS

- slant culture of avirulent strain of *Corynebacterium diphtheriae*
- methylene blue (Loeffler's)
- wash bottle
- bibulous paper

After examining the slide, compare it with the photomicrograph in illustration 1, figure 14.4 (page 103) Record your observations on Laboratory Report 10–13.



(1) A bacterial smear is stained with methylene blue for one minute.

(2) Stain is briefly washed off slide with water.

(3) Water drops are carefully blotted off slide with bibulous paper.

FIGURE 11.1 Procedure for simple staining

Negative Staining

12

EXERCISE

Another stain that can be used to study the morphology of bacterial cells is the **negative stain.** Negative stains such as nigrosin and india ink do not penetrate the bacterial cell but rather cause the background area around a cell to be opaque or dark. Cells appear as transparent objects against the dark background. The negative stain reveals the shape of the cell and extracellular features such as capsules. The method consists of mixing the organism with a small amount of stain and spreading a very thin film over the surface of the slide. For the negative stain, cells are not heat fixed prior to the application of the negative stain.

The method can be useful for determining cell morphology and size of the cells. Because no heat fixation was performed, no shrinkage of the cells has occurred and size determinations are more accurate than those determined on fixed material. Avoiding heat fixation is also important if the capsule surrounding bacterial cells is to be observed because heating severely shrinks this structure. The negative stain is also useful for observing spirochaetes that do not stain readily with ordinary dyes.

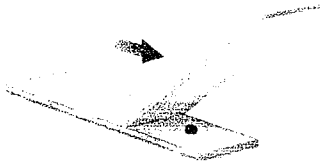
THREE METHODS

Negative staining can be done by one of three different methods. Figure 12.1 illustrates the more commonly used method in which the organisms are mixed in a drop of nigrosin and spread over the slide with another slide. The goal is to produce a smear that is thick at one end and feather-thin at the other end. Somewhere between the too thick and too thin areas will be an ideal spot to study the organisms.

Figure 12.2 illustrates a second method, in which organisms are mixed in only a loopful of nigrosin instead of a full drop. In this method, the organisms are spread over a smaller area in the center of the slide with an inoculating needle. No spreader slide is used in this method.

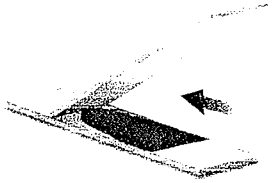
The third procedure (Woeste-Demchick's method), which is not illustrated here, involves applying ink to a conventional smear with a black felt-tip marking pen. If this method is used, it should be done on a smear prepared in the manner described in exercise 13. Simply put, the technique involves applying a *single coat* of marking-pen ink over a smear.

(1) Organisms are dispersed into a small drop of nigrosin or india ink. Drop should not exceed ⅛" diameter and should be near the end of the slide.



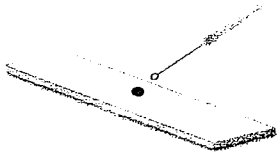
(2) Spreader slide is moved toward drop of suspension until it contacts the drop causing the liquid to be spread along it's spreading edge.

(3) Once spreader slide contacts the drop on the bottom slide, the suspension will spread out along the spreading edge as shown.

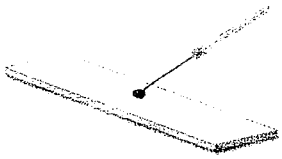


(4) Spreader slide is pushed to the left, dragging the suspension over the bottom slide. After the slide has air-dried, it may be examined under oil immersion.

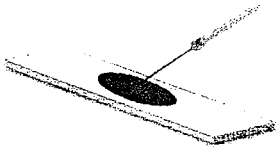
FIGURE 12.1 Negative staining technique, using a spreader slide



(1) A loopful of nigrosin or india ink is placed in the center of a clean microscope slide.



(2) A sterile inoculating wire is used to transfer the organisms to the liquid and mix the organisms into the stain.



(3) Suspension of bacteria is spread evenly over an area of one or two centimeters with the straight wire.

(4) Once the preparation has completely air-dried, it can be examined under oil immersion. No heat should be used to hasten drying.

FIGURE 12.2 A second method for negative staining

Note in the procedure below that slides may be made from organisms between your teeth or from specific bacterial cultures. Your instructor will indicate which method or methods you should use and demonstrate some basic aseptic techniques. Various options are provided here to ensure success.

MATERIALS

- microscope slides (with polished edges)
- nigrosin solution or india ink
- slant cultures of *S. aureus* and *B. megaterium*
- inoculating straight wire and loop
- sterile toothpicks
- Bunsen burner
- Sharpie marking pen
- felt-tip marking pen (see Instructor's Handbook)

1. Swab down your tabletop with disinfectant in preparation for making slides.
2. Clean two or three microscope slides with Bon Ami to rid them of all dirt and grease.
3. By referring to figure 12.1 or 12.2, place the proper amount of stain on the slide.
4. **Oral Organisms:** Remove a small amount of material from between your teeth with a sterile straight toothpick and mix it into the stain on the slide. Be sure to break up any clumps of organisms with the toothpick or a sterile inoculating loop. When using a loop, *be sure to flame it first to make it sterile.*

> **CAUTION** If you use a toothpick, discard it into a beaker of disinfectant.

5. **From Cultures:** With a *sterile* straight wire, transfer a very small amount of bacteria from the slant to the center of the stain on the slide.
6. Spread the mixture over the slide according to the procedure used in figure 12.1 or 12.2.
7. Allow the slide to air-dry and examine with an oil immersion objective.

LABORATORY REPORT

Draw a few representative types of organisms on Laboratory Report 10–13. If the slide is of oral organisms, look for yeasts and hyphae as well as bacteria. Spirochaetes may also be present.

15

Capsular Staining

13

EXERCISE

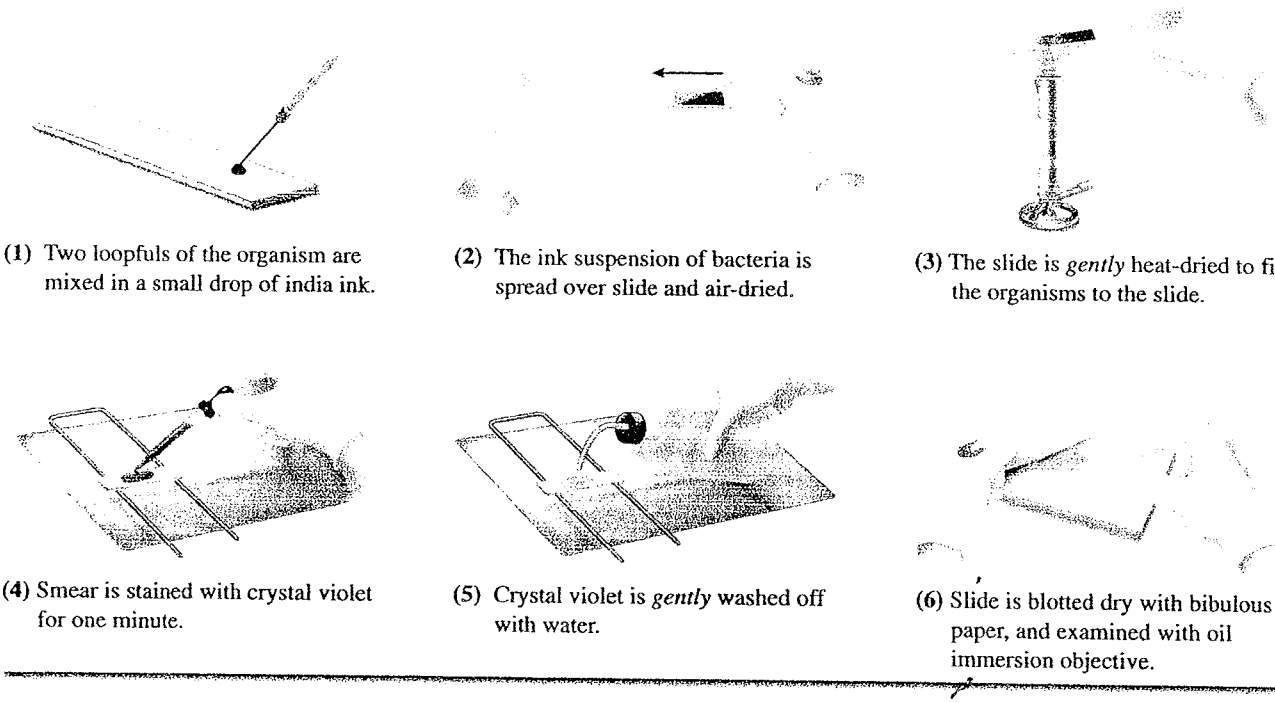
Some bacterial cells are surrounded by an extracellular slime layer called a **capsule** or **glycocalyx.** This structure can play a protective role for certain pathogenic bacteria such as *Streptococcus pneumoniae.* The capsule prevents phagocytic white blood cells from engulfing and destroying this bacterial pathogen, enabling the organism to invade the lungs and cause pneumonia. The capsule is also a means for many bacteria to attach to solid surfaces in the environment. For example, *Streptococcus mutans* can attach to the surface of a tooth by its capsular material resulting in the formation of dental plaque, which contributes to the process of tooth decay in humans. Most capsules are usually composed of polysaccharides but in some cases a capsule can consist of polypeptides with unique amino acids. Evidence supports the view that probably all bacterial cells have some amount of slime layer, but in most cases the amount is not enough to be readily discernible.

Staining of the bacterial capsule cannot be accomplished by ordinary staining procedures. If smears are heat fixed prior to staining, the capsule shrinks or is destroyed and cannot be seen in stains. If the cells are not heat fixed to the slide, they can wash off during washing procedures. However, the capsule can be demonstrated by combining the methods for the simple stain and the negative stain as shown in figure 13.1. You will use this method to stain the capsule of *Klebsiella pneumoniae.*

MATERIALS

- 36–48 hour milk culture of *Klebsiella pneumoniae*
- india ink
- crystal violet

Observation Examine the slide under oil immersion and compare your slide with illustration 2, figure 14.4 on page 103. Record your results on Laboratory Report 10–13.



(1) Two loopfuls of the organism are mixed in a small drop of india ink.

(2) The ink suspension of bacteria is spread over slide and air-dried.

(3) The slide is *gently* heat-dried to fix the organisms to the slide.

(4) Smear is stained with crystal violet for one minute.

(5) Crystal violet is *gently* washed off with water.

(6) Slide is blotted dry with bibulous paper, and examined with oil immersion objective.

FIGURE 13.1 Procedure for demonstration of a capsule

Gram Staining

14

EXERCISE

In 1884, the Danish physician Christian Gram was trying to develop a staining procedure that would differentiate bacterial cells from eukaryotic nuclei in stained tissue samples. Although Gram was not completely successful in developing a tissue stain, what resulted from his work is the most important stain in bacteriology, the Gram stain. This technique separates bacteria into two groups: gram-positive and gram-negative bacteria. The procedure is based on the fact that gram-positive bacteria retain a crystal violet-iodine complex through decolorization with alcohol or acetone. Gram-positive bacteria appear as purple when viewed by microscopy. In contrast, alcohol or acetone removes the crystal violet-iodine complex from gram-negative bacteria. These bacteria must, therefore, be counterstained with a red dye, safranin, after the decolorization step in order to be visualized by microscopy. Hence, gram-negative bacteria appear as red cells when viewed by microscopy.

Figure 14.1 illustrates the appearance of cells after each step in the Gram-stain procedure. Note that initially both gram-positive and gram-negative cells are stained by the **primary stain,** crystal violet. In the second step of the procedure, Gram's iodine is added to the smear. Iodine is a **mordant** that complexes with the crystal violet and forms an insoluble complex in gram-positive cells. At this point, both types of cells will still appear as purple. The dye-mordant complex is not removed from gram-positive bacteria but is leached from gram-negative cells by the alcohol or acetone in the **decolorization** step. After decolorization, gram-positive cells are purple but gram-negative cells are colorless. In the final step, a **counterstain,** safranin, is applied, which stains the colorless gram-negative cells. The appearance of the gram-positive cells is unchanged because the crystal violet is a much more intense stain than safranin.

Reagent	Gram positive	Gram negative
None (Heat-fixed cells)		
Crystal Violet (20 seconds)		
Gram's Iodine (1 minute)		
Ethyl Alcohol (10–20 seconds)		
Safranin (1 minute)		

FIGURE 14.1 Color changes that occur at each step in the gram-staining process

The mechanism for how the Gram stain works is related to chemical differences in the cell walls of gram-positive and gram-negative bacteria (figure 14.2). When viewed by electron microscopy, gram-positive cells have a thick layer of **peptidoglycan** that comprises the cell wall of these organisms. In contrast, the cell wall in gram-negative cells consists

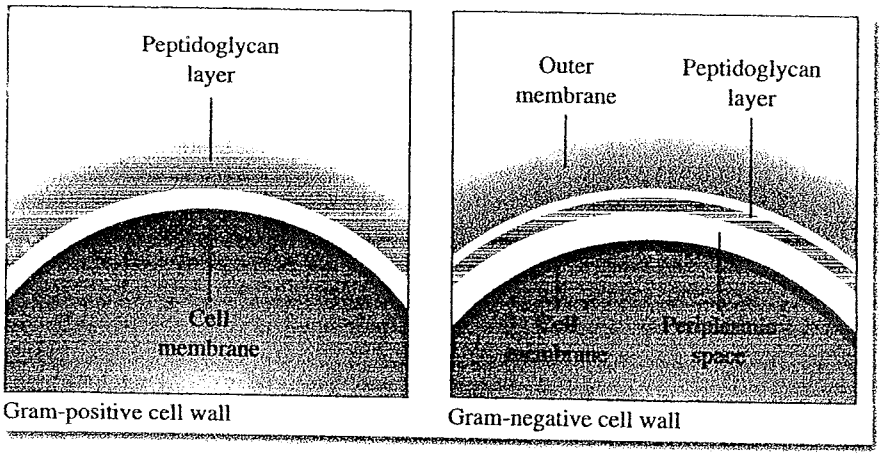


FIGURE 14.2 Comparison of gram-positive and gram-negative cell walls

of an outer membrane that covers a much thinner layer of peptidoglycan. It is these significant differences in structure that probably determines whether the dye-mordant complex is removed from the gram-negative cell or remains associated with the gram-positive cell.

Of all the staining techniques you will use in microbiology, the Gram stain is one of the most important. It will be critical in identifying your unknown bacteria and you will use it routinely in many exercises in this manual. Although this technique seems quite simple, performing it with a high degree of reliability requires some practice and experience. Several factors can affect the outcome of the procedure:

1. It is important to use cultures that are 16–18 hours old. Gram-positive cultures older than this can convert to gram-variable or gram-negative and give erroneous results. (It is important to note that gram-negative bacteria never convert to gram-positive.)
2. It is critical to prepare thin smears. Thin smears allow the observation of individual cells and any arrangement in which the cells occur. Furthermore, the thickness of your smears can affect decolorization. Thick smears can entrap the primary stain, which is not removed by alcohol or acetone. Cells that occur in the entrapped stain can appear gram-positive leading to erroneous results.
3. Decolorization is the most critical step in the Gram-stain procedure. If the destaining reagent is overapplied, the dye-mordant complex can eventually be removed from gram-positive cells, converting them to gram-negative cells.

During this laboratory period, you will be provided an opportunity to stain several different kinds of bacteria to see if you can achieve the degree of success that is required. Remember, if you don't master this technique now, you will have difficulty with your unknowns later.

STAINING PROCEDURE

MATERIALS

- slides with heat-fixed smears
- Gram-staining kit and wash bottle
- bibulous paper

1. Cover the smear with **crystal violet** and let stand for *20 seconds* (see figure 14.3).

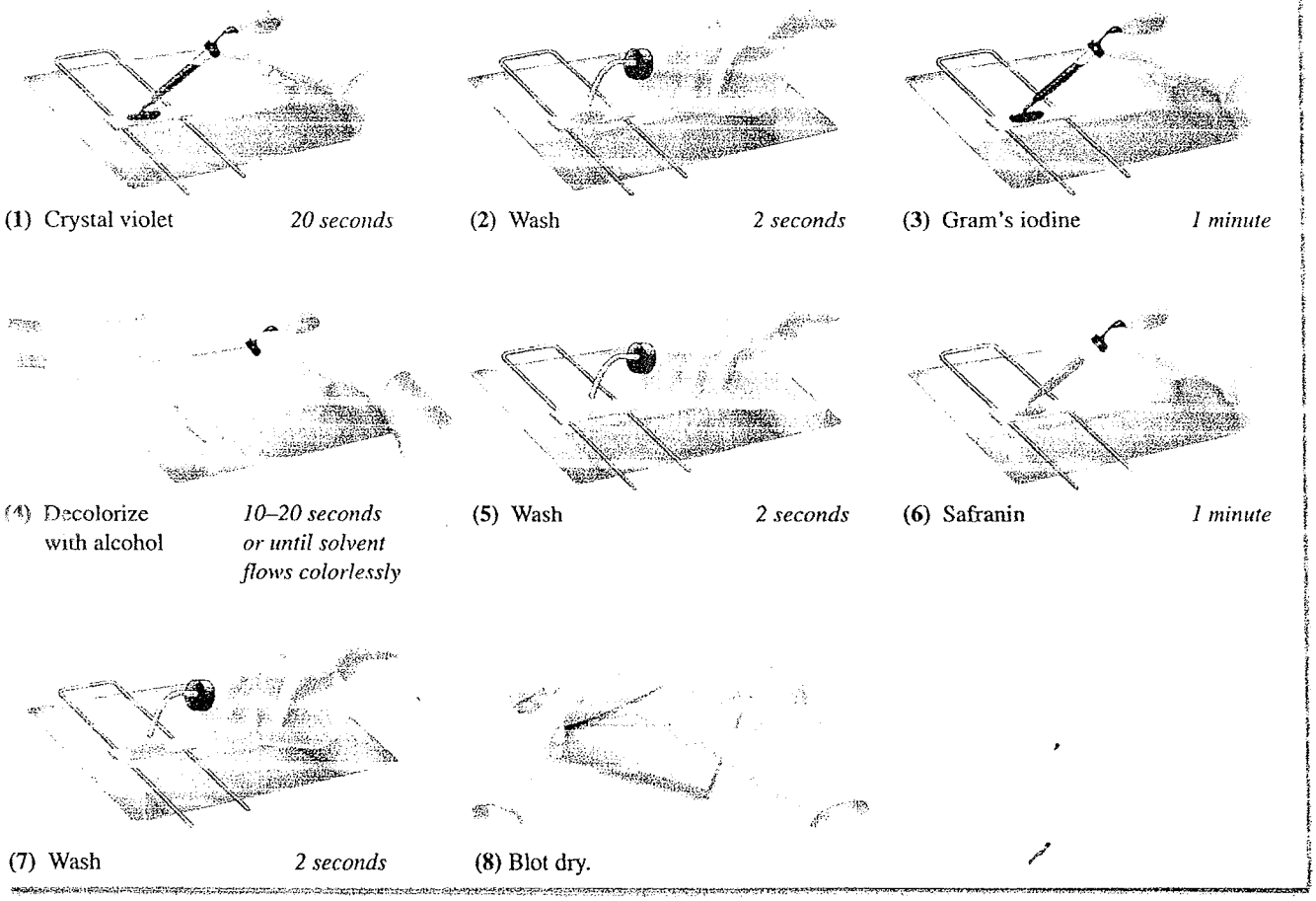


FIGURE 14.3 The Gram-staining procedure

18

2. Briefly wash off the stain, using a wash bottle of distilled water. Drain off excess water.
3. Cover the smear with **Gram's iodine** solution and let it stand for *one minute.* (Your instructor may prefer only 30 seconds for this step.)
4. Wash off the Gram's iodine. Hold the slide at a 45-degree angle and allow the 95% alcohol to flow down the surface of the slide. Do this until the alcohol is colorless as it flows from the smear down the surface of the slide. *This should take no more than 20 seconds for properly prepared smears.* Note: thick smears can take longer than 20 seconds for decolorization.
5. Stop decolorization by washing the slide with a gentle stream of water.
6. Cover the smear with **safranin** for 1 minute.
7. Wash gently for a few seconds, blot dry with bibulous paper, and air-dry.
8. Examine the slide under oil immersion.

ASSIGNMENTS

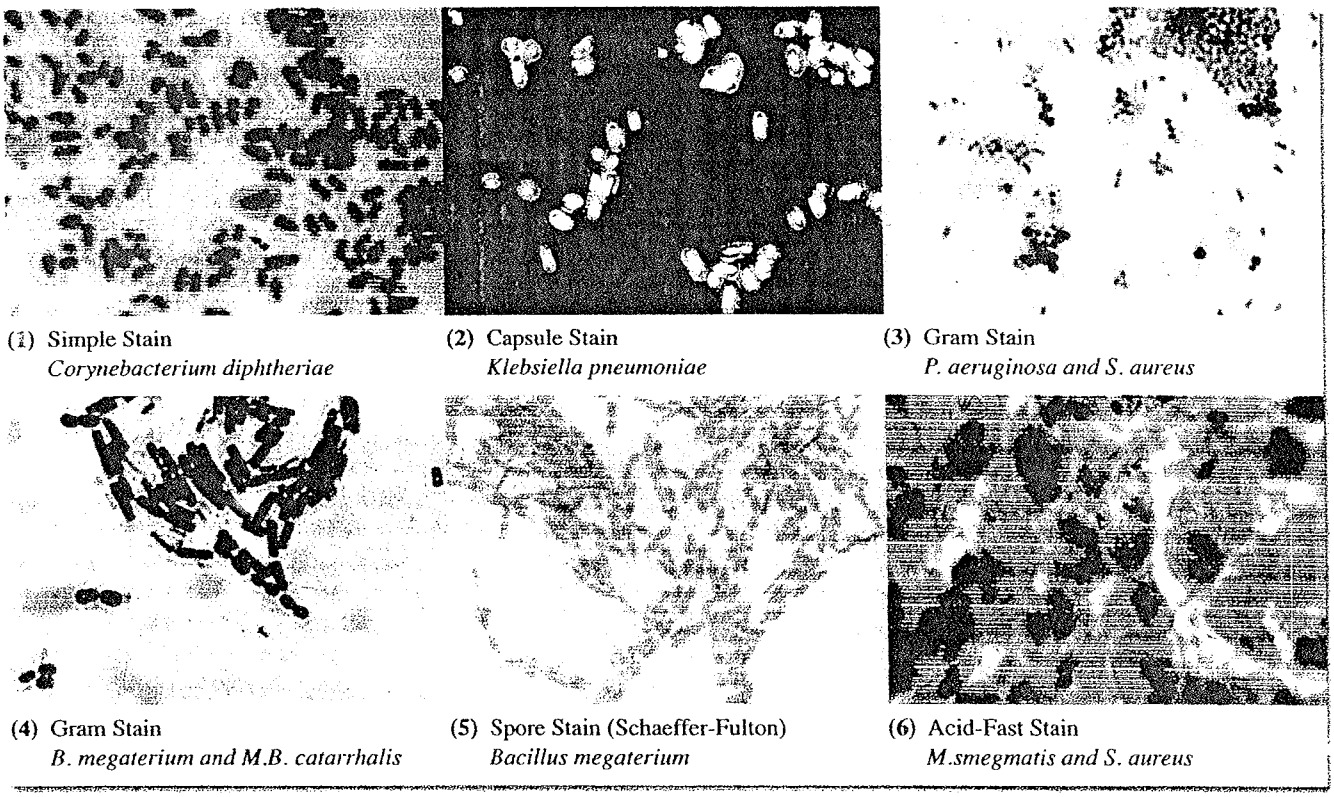
The organisms that will be used here for Gram staining represent a diversity of form and staining characteristics. Some of the rods and cocci are gram-positive; others are gram-negative. One rod-shaped organism is a spore-former and another is acid-fast. The challenge here is to make Gram-stained slides of various combinations that reveal their differences.

MATERIALS

- broth cultures of *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Moraxella (Branhamella) catarrhalis*
- nutrient agar slant cultures of *Bacillus megaterium* and *Mycobacterium smegmatis*

Mixed Organisms ! (Triple Smear Practice Slides) Prepare three slides with three smears on each slide. On the left portion of each slide make a smear of *Staphylococcus aureus.* On the right portion of each slide make a smear of *Pseudomonas aeruginosa.* In the middle of the slide make a smear that is a mixture of both organisms, using two loopfuls of each organism. ***Be sure to flame the loop sufficiently to avoid contaminating cultures.***

Gram stain one slide first, saving the other two for later. Examine the center smear. If done properly, you should see purple cocci and pink rods as shown in illustration 3, figure 14.4.



(1) Simple Stain
Corynebacterium diphtheriae

(2) Capsule Stain
Klebsiella pneumoniae

(3) Gram Stain
P. aeruginosa and S. aureus

(4) Gram Stain
B. megaterium and M.B. catarrhalis

(5) Spore Stain (Schaeffer-Fulton)
Bacillus megaterium

(6) Acid-Fast Stain
M.smegmatis and S. aureus

FIGURE 14.4 Photomicrographs of representative staining techniques (8000x)
(2) © Science VU/Visuals Unlimited (5) Courtesy of Lansing Prescott (6) © Science VU/Visuals Unlimited

19

Call your instructor over to evaluate your slide. If the slide is improperly stained, the instructor will be able to tell what went wrong by examining all three smears. He or she will inform you how to correct your technique when you stain the next triple smear reserve slide.

Record your results on Laboratory Report 14–16 by drawing a few cells in the appropriate circle.

Mixed Organisms II Make a Gram-stained slide of a mixture of *Bacillus megaterium* and *Moraxella (Branhamella) catarrhalis.*

This mixture differs from the previous slide in that the rods (*B. megaterium*) will be purple and the cocci (*M.B. catarrhalis*) will be large pink diplococci. See illustration 4, figure 14.4.

As you examine this slide, look for clear areas in the rods, which represent endospores. Since endospores are refractile and impermeable to crystal violet, they will appear as transparent holes in the cells.

Draw a few cells in the appropriate circle on your Laboratory Report sheet.

Acid-Fast Bacteria To see how acid-fast mycobacteria react to Gram's stain, make a Gram-stained slide of *Mycobacterium smegmatis.* If your staining technique is correct, the organisms should appear gram-positive.

Draw a few cells in the appropriate circle on your Laboratory Report sheet.

Spore Staining:
Two Methods

15
EXERCISE

When species of bacteria belonging to the genera *Bacillus* and *Clostridia* exhaust essential nutrients, they undergo a complex developmental cycle that produces resting stages called **endospores.** Endospores allow these bacteria to survive environmental conditions that are not favorable for growth. If nutrients once again become available, the endospore can go through the process of germination to form a new vegetative cell and growth will resume. Endospores are very dehydrated structures that are not actively metabolizing. Furthermore, they are resistant to heat, radiation, acids, and many chemicals, such as disinfectants, that normally harm or kill vegetative cells. Their resistance is due in part to the fact that they have a thick protein coat, or **exosporium,** that forms a protective barrier around the spore. Heat resistance is associated with the water content of endospores. The higher the water content of an endospore, the less heat resistant the endospore will be. During sporulation, the water content of the endospore is reduced to 10–30% of the vegetative cell. This results because calcium ions complex with spore-specific proteins and a chemical, dipicolinic acid. The latter compound is not found in vegetative cells. This complex forms a gel that controls the amount of water that can enter the endospore, thus maintaining its dehydrated state.

Since endospores are not easily destroyed by heat or chemicals, they define the conditions necessary to establish sterility. For example, to destroy endospores by heating, they must be exposed for 15 to 20 minutes to steam under pressure, which generates temperatures of 121°C. Such conditions are produced in an **autoclave.**

The resistant properties of endospores also mean that they are not easily penetrated by stains. For example in exercise 14, you observed that endospores did not readily Gram stain. If endospore-containing cells are stained by basic stains such as crystal violet, the spores appear as unstained areas in the vegetative cell. However, if heat is applied while staining with malachite green, the stain penetrates the endospore and becomes entrapped in the endospore. The malachite green is not removed by subsequent washing with decolorizing agents or water. In this instance, heat is acting as mordant to facilitate the uptake of the stain.

SCHAEFFER-FULTON METHOD

The Schaeffer-Fulton method, which is depicted in figure 15.1, utilizes malachite green to stain the endospore and safranin to stain the vegetative portion of the cell. Utilizing this technique, a properly stained spore-former will have a green endospore contained in a pink sporangium. Illustration 5, figure 14.4, on page 103 reveals what such a slide looks like under oil immersion.

After preparing a smear of *Bacillus megaterium,* follow the steps outlined in figure 15.1 to stain the spores.

MATERIALS

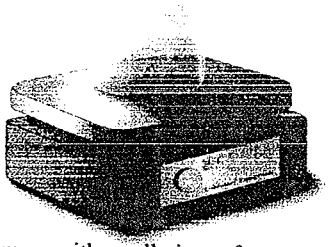
- 24–36 hour nutrient agar slant culture of *Bacillus megaterium*
- electric hot plate and small beaker (25 ml)
- spore-staining kit consisting of a bottle each of 5% malachite green and safranin

DORNER METHOD

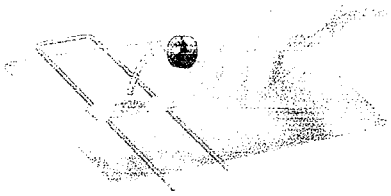
The Dorner method for staining endospores produces a red spore within a colorless sporangium. Nigrosin is used to provide a dark background for contrast. The six steps involved in this technique are shown in figure 15.2. Although both the sporangium and endospore are stained during boiling in step 3, the sporangium is decolorized by the diffusion of safranin molecules into the nigrosine.

Prepare a slide of *Bacillus megaterium* that utilizes the Dorner method. Follow the steps in figure 15.2.

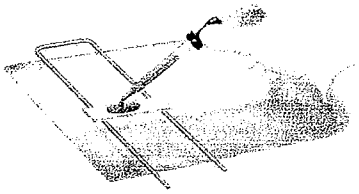
21



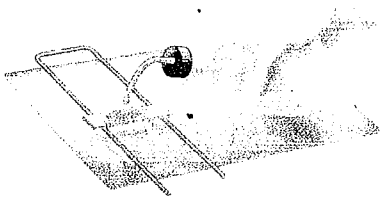
(1) Cover smear with small piece of paper toweling and saturate it with malachite green. Steam over boiling water for *5 minutes*. Add additional stain if stain boils off.



(2) After the slide has cooled sufficiently, remove the paper toweling and rinse with water for 30 seconds.



(3) Counterstain with safranin for about *20 seconds*.



(4) Rinse briefly with water to remove safranin.

(5) Blot dry with bibulous paper, and examine slide under oil immersion.

FIGURE 15.1 The Schaeffer-Fulton spore stain method

MATERIALS

- nigrosin
- electric hot plate and small beaker (25 ml)
- small test tube (10 × 75 mm size)
- test-tube holder
- 24–36 hour nutrient agar slant culture of *Bacillus megaterium*

QUICK SPORE STAIN

A variation on the Schaeffer-Fulton method is a quick method that uses the same stains.

MATERIALS

- *Bacillus subtilis* slant cultures, older than 36 hours
- malachite green stain
- safranin stain
- staining racks
- clothespins

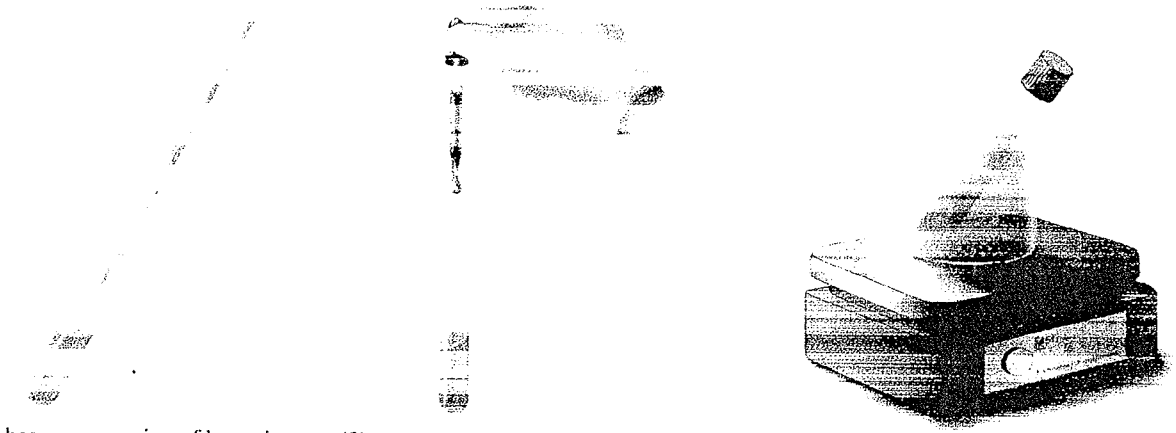
PROCEDURE

1. Prepare a smear of the organism and allow it to air-dry.
2. Grasp the slide with the air-dried smear with a clothespin and pass it through a Bunsen burner flame 10 times. Be careful not to overdo the heating as the slide can break.
3. Immediately flood the smear with malachite green and allow to stand for 5 minutes.
4. Wash the smear with a gentle stream of water.
5. Stain with safranin for 45 seconds. Spores will be green and the vegetative cell will be red.

LABORATORY REPORT

After examining the organisms under oil immersion, draw a few cells in the appropriate circles in Laboratory Report 14–16.

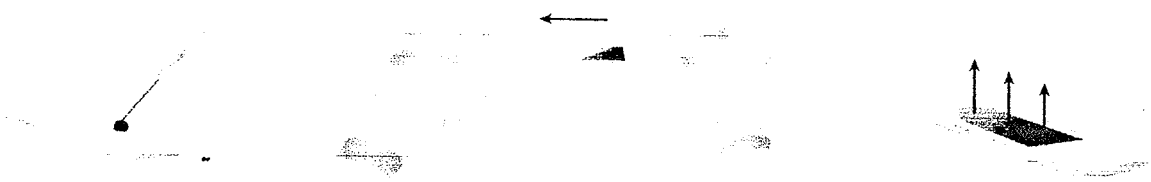
22



(1) Make a heavy suspension of bacteria by dispersing several loopfuls of bacteria in 5 drops of sterile water.

(2) Add 5 drops of carbolfuchsin to the bacterial suspension.

(3) Heat the carbolfuchsin suspension of bacteria in a beaker of boiling water for *10 minutes*.



(4) Mix several loopfuls of bacteria in a drop of nigrosin on the slide.

(5) Spread the nigrosin-bacteria mixture on the slide in the same manner as in Exercise 11 (Negative Staining).

(6) Allow the smear to air-dry. Examine the slide under oil immersion.

FIGURE 15.2 **The Dorner spore stain method**

23

Acid-Fast Staining:
Ziehl-Neelsen Method

EXERCISE 16

Bacteria in the genus *Mycobacterium* and some in the genus *Nocardia* contain a waxy material in their cell walls called **mycolic acid.** This material significantly affects the staining properties of these organisms and prevents them from being stained by many of the stains used in microbiology. However, if they are stained with carbolfuchsin and heat is applied during the staining procedure, the carbolfuchsin is able to penetrate the cell and it is not removed by subsequent washing with acid-alcohol. Such bacteria are said to be **acid-fast** and appear pink or red in stained smears. This property sets them apart from most other bacteria, which are decolorized by the acid-alcohol and must be counterstained with methylene blue to be seen. In the acid-fast stain, heat is acting as a mordant to soften the mycolic acid so the stain can penetrate the cell.

The acid-fast stain is an important diagnostic tool in the identification of *Mycobacterium tuberculosis* the causative agent of tuberculosis, and *Mycobacterium leprae,* the bacterium that causes leprosy in humans. When the stain is used in the diagnosis of these diseases, the mycobacteria appear as red rods whereas tissue cells and non-acid-fast bacteria are stained blue. An example of an acid-fast stain is seen in photo 6 in figure 14.4.

In the following exercise, you will prepare an acid-fast stain of a mixture of *Mycobacterium smegmatis* and *Staphylococcus aureus. M. smegmatis* is a nonpathogenic acid-fast rod that occurs in soil and on the external genitalia of humans. *S. aureus* is a non-acid-fast coccus that can also be part of the normal flora of humans as well as a potential pathogen.

MATERIALS

- nutrient agar slant culture of *Mycobacterium smegmatis* (48-hour culture)
- nutrient broth culture of *S. aureus*
- electric hot plate and small beaker
- acid-fast staining kit (carbolfuchsin, acid-alcohol, and methylene blue)

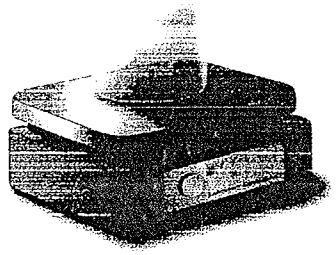
Smear Preparation Prepare a mixed culture smear by placing two loopfuls of *S. aureus* on a slide and transferring a small amount of *M. smegmatis* to the broth on the slide with an inoculating needle. Since the smegma bacilli are waxy and tend to cling to each other in clumps, break up the masses of organisms with the inoculating needle. After air-drying the smear, heat-fix it.

Staining Follow the staining procedure outlined in figure 16.1.

Examination Examine under oil immersion and compare your slide with illustration 6, figure 14.4.

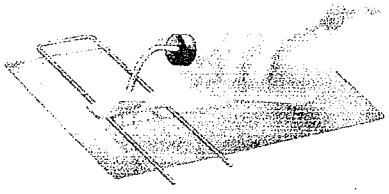
Laboratory Report Record your results in Laboratory Report 14–16.

24

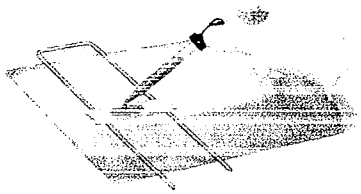


(1) Cover smear with carbolfuchsin. Steam over boiling water *5 minutes*. Add additional stain if stain boils off.

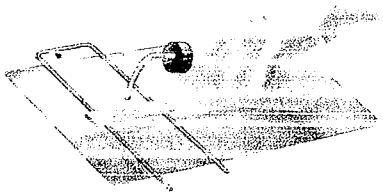
(2) After slide has cooled, decolorize with acid-alcohol for *15–20 seconds*.



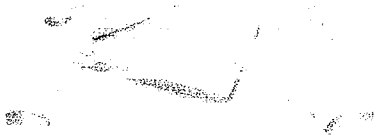
(3) Stop decolorization action of acid-alcohol by rinsing *briefly* with water.



(4) Counterstain with methylene blue for *30 seconds*.



(5) Rinse *briefly* with water to remove excess methylene blue.



(6) Blot dry with bibulous paper. Examine directly under oil immersion.

FIGURE 16.1 **Ziehl-Neelsen acid-fast staining procedure**

25

Lab - 1

TISSUE PROCESSING

* Histology:

It is the branch of science which deals with the gross &microscopic study of normal tissue

*Histopathology:

It is the branch of science which deals with the gross & microscopic study of tissue affected by disease.

Histotechniquse :

The techniques for processing the tissue , whether biopsies,larger specimen removed at surgery, or tissue from autopsy so as at to enable the pathologist to study them under the microscope .

Protocols followed in Histotechniquse

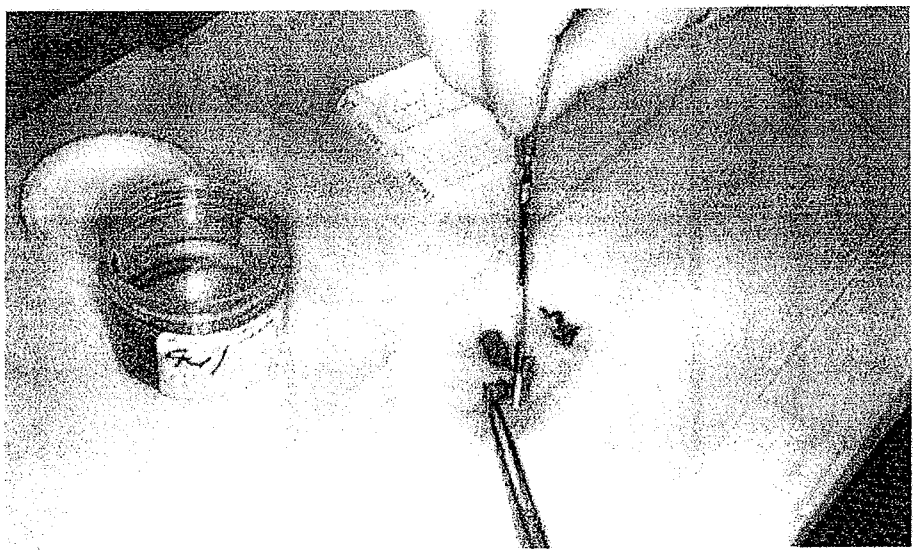
1- Recept & Identification
2- labeling of the specimen with numbering.
3- fixation .
4- Washing .
5- dehydration .
6- clearing .
7- impregnation .
8- Embedding :
9- section cutting .
10- staining .
11- mounting .

Lab-2

protocols followed in Histotechniquse

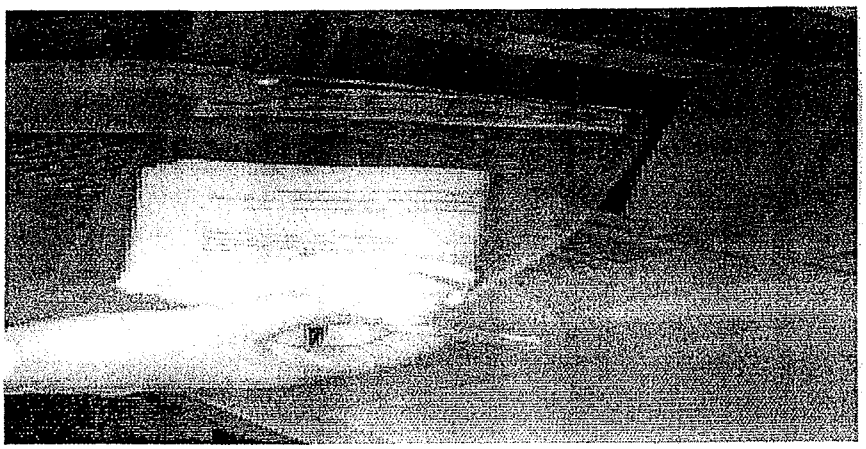
1- Recept & Identification •

Tissue specimen received in the surgical pathology laboratory have a request form that list the patient information and history along with a description of the site of origin .



2-labeling of the specimen with numbering.

The specimen are accessioned by giving them a number that will identify each specimen for each patient .



3- fixation .

* It is a process in which aspecimen is treated by exposing it to afixative for aparticular period of time in order to facilitate the succeeding step .

* The puropose of fixation is to preserve tissue permanently in as life-like astate as possible .

• The fixative should be 15-20 times more in volume then the specimen

• Mechanism of action . it forms cross linke between amino acide of proteins thereby making them insoluble .

• The bite should of size of approximately 2x2 cm & 4-6 micrometer in thickness for optimum fixation to take place .

• Tiny biopsies or small specimen can be wrapped in filter paper and then put in a cassette & fixed .

Aim of fixation

1- It should prevent autolysis & putrefaction of the cell .
2- It should penetrate evenly and rapidly .
3- It should harden the tissues .
4- Increase the optical density .
5- Should not cause shrinkage or swelling of the cells .
6- Must not react with the receptor sites & thus must not interefere with the staining procedure .
7- It must be cheap and easily avaible .

Properties of an Ideal fixative

1- Prevents autolysis and bacterial decomposition .

2-Preserves tissue in their natural state and fix all components .

3- Make the cellular components insoluble to reagent used in tissue processing .

4- Preserves tissue volume .

5- Avoid excessive hardness of tissue .

6- Allows enhanced staining of tissue .

7- Should be non toxic and non-allergic for user .

8- should not be very expensive .

Classification of fixatives

A) Physical fixatives : heat , freezing ..... etc .

B) Chemical fixatives : 1- simple fixatives .

2- compound fixative .

Chemical Fixatives

Simple Fixatives

- ✓ Formaline
- ✓ Mercuric chloride
- ✓ Osmic acid
- ✓ Picric acid
- ✓ Acetone
- ✓ Ethyle alchohol
- ✓ Osmium tetroxide
- ✓ Osmic acid

Compound Fixatives

Microanatomical	Cytological	Histochemical
Formal Saline		Cold acetone
Neutral buffer Formaline		Ethanol
Zenker's fluid		
Bouin's fluid		

Cytological

Nuclear	Cytoplasmic
Carnoy's Fluid	Champy's Fluid

1-simple fixatives :

The most commonly used fixative is Formalin . it is prepared by mixing 40% formaldehyde gas in 100 w/v of distilled water . the resultant mixture is 100% formalin .routinely , 10% formalin is used which is prepared by mixing 10 ml of 100% formalin in 90 ml of distilled water .

Other simple fixative :

1-Picric acid .

2-Osmic acid .

3- Mercuric chloride.

ADVANTAGES FORMALIN	DIS ADVANTAGES FORMALIN
1- Rapid penetration 2- Easy availability&cheap 3- Dose not overharden tissue 4- Fix lipids for frozen section 5- Ideal for mailing	1-Irritant to the nose ,the eyes and mucous membranes . 2- Formation of precipitate of para – formaldehyde which can be prevented by adding 11-16% methanol . 3- Formation of black formalin pigment ,Acid formaldehyde hematin.

Lab- 3

2- Compound fixatives :

- a) Microanatomical fixatives :these are used to preserve the anatomy of the tissue like ,10%formalin saline, Zenker's fluid ,Bouin's fluid .

- b) Cytological fixatives : these are used to fix intracellular structures it two type

Nuclear fixative	Cytoplasmic fixative
Carnoy's fluid , Clarke's fluid	Champy's fluid , Regaud's fluid

- C) Histochemical fixatives :These are used to demonstrate the chemical constituents of the cell like Cold acetone , Ethanol

5- Dehydration .

It is the process in which the water content in the tissue to be processed is completely reduced by passing the tissue through increasing concentration of dehydrating agents .

The various dehydration agents used are : Ethyl alcohol , Acetone , Isopropyl alcohol ,Dioxane .

The duration of the procedure can be noted down as :

1-30% alcohol – 1 hour .
2- 50% alcohol – 1 hour.
3-70% alcohol – 1 hour .
4-70% alcohol – 1 hour
5- 90% alcohol – 1 hour .
6- 95 % alcohol – 1 hour .

7-Absolute alcohol – 1 hour .

8- Absolute alcohol – 1 hour .

Dehydration is done so that the wax i.e Paraffin wax , which is used for impregnation, can be easily miscible as it is immiscible with water.

6- Clearing (Dealcoholization) .

It is the procedure where in the alcohol in the tissue is replaced by fluid which will dissolve the wax used for impregnating the tissues .

The various clearing agents used are :

* Cedar wood oil : the best agent but is expensive .

* Benzene : it is carcinogenic .

* Xylene : it is most commonly used .

* chloroform : tixic and expensive .

7- Impregnation :

In this the tissue is kept in a wax bath containing molten paraffin wax foe 6-8 hours .the wax is infiltrated in the interices of the tissue which increases the optical differentiation & hardens the tissue & helps in easy sectioning of the tissue .

The various waxes which used are : (paraffin wax ,paraplast , Gelatin)

8- Embedding :

It is done by transferring the tissue which has been cleared of the alcohol to a mould filled with molten wax & is allowed to cool & solidify .after solidification , awax block is obtained which is then sectioned to obtain ribbons .

* Type of moulds :

A) Leuckhart's Moulds : L-shaped brass pieces which is placed in opposing positions & can be manipulated to increase or decrease the size of the block be prepared .

B) Glass or Metal petri dishes

C) Watch glass .

D) Paper boats .

9- Section cutting

It is the procedure in which the blocks which have been prepared are cut or sectioned and thin strips of varying thickness are prepared .

The instrument by which this is done is called as a Microtome

Type of Microtome : sliding , rotary , rocking , freezing , base sledge

Rotary microtome :

It is the most commonly used . Also known as Minnot s Rotary microtom

In this the Blok holder moves up and down while the knife remains fixed

It is suitable for cutting of small tissues & serial sections can be taken on it.

Parts of a Microtome (Rotary)

A. Block holder .

B. Knife clamp screws

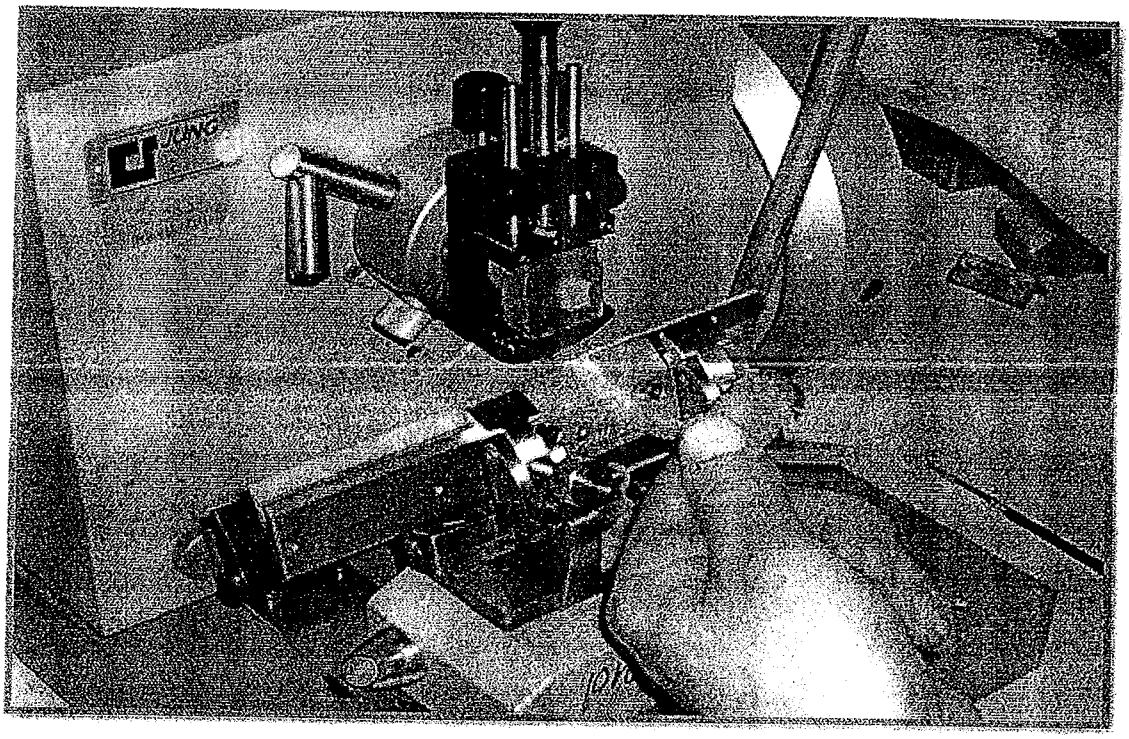
C. Knife clamps

D. Block adjustment

E. Thickness gauge

F. Angle of tilt adjustment

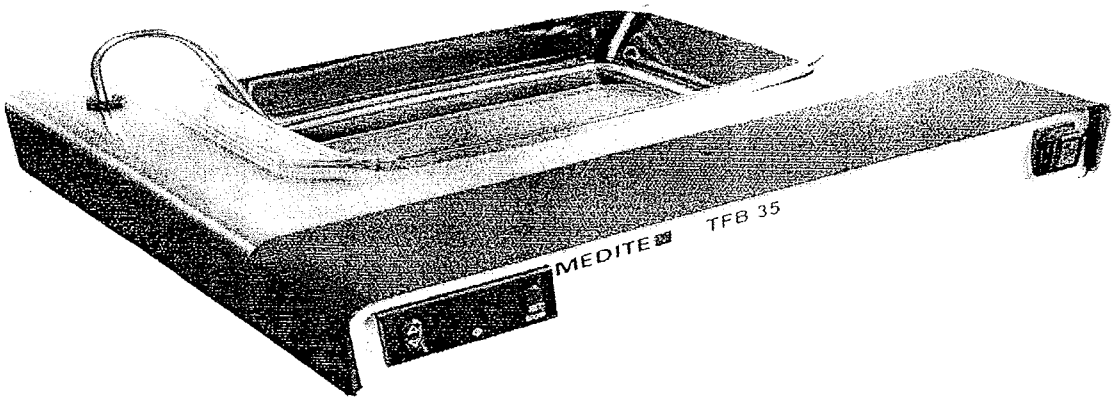
G. Operating handle



Tissue floatation bath

It is thermostatically controlled water both with the inside colored black.

It is maintained at a temperature maintained 5-6 degree paraffin wax.



10 - Deparaffinization and Rehydration :

To color or stain the tissue sections , the paraffin must be dissolved out , again with xylene and the slide must then be rehydrated through a series of solutions of descending alcohol concentration . Because paraffin section are colorless .

* 100 % alcohol – 1 hour .
* 90 % alcohol – 1 hour.
* 80 % alcohol – 1 hour .
* 70 % alcohol – 1 hour
50 % alcohol – 1 hour .

11- Staining :

Is process by which we give colour to asection , there are hundred of stains available , and can be classification to : Acid stains , Basic stains , Neutral stains .

Acid stains :In an acid dye the basic component is colored and the acid component is colorless , Acid dyes stain basic components e.g. eosin stains cytoplasm , the color imparted is shade of red .

Basic stains : In an basic dye the acide component is colored and the basic component is colorless , Basic dyes stain acidic components e.g. basic fuchsin stains nucleus , thc color imparted is shade of blue .

Neutral stains : When an acid dye is combined with abasic dye aneutral dye is formed , As it contains both colored radicals , it gives different colors to cytoplasm and nucleus simultaneously .this is the basis of Leishman stain .

12- Mounting :

Adhesives used for fixing the sections on the slides , the adhesives like : Albumin solution(mayor's egg albumin) , Starch paste , Gelatin .

Mountants permanent agent : Canada balsam , Dpx , Terpene resin ,