Microbiology Biology Lab Manual

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Preface

In the area of microbiology, laboratory is the most important learning center where student gets to see, touch, perform lab activities, and otherwise experience much of the information explained in lecture portion of the course. Medical and fundamental microbiology is an exceptionally great but broad discipline as diverse as biotechnology, biochemistry, cell biology, physiology, genetics, taxonomy, pathogenic bacteriology, food and industrial microbiology, and ecology. The person who studies about the microbiology is known as microbiologist. Microbiologist must be acquainted with several biological disciplines and with all major groups of microorganisms such as viruses, bacteria, fungi, algae, and protozoa. Students may be new to the subject need an introduction to the whole before concentrating on those parts of greatest interest to them. I believe that this lab manual provides a balanced introduction to all major areas of microbiology for a variety of students.

This lab manual is designed with orientations ranging from basic microbiology to medical and applied microbiology. Of course, students preparing for their careers in biotechnology, medicine, dentistry, nursing, and allied health professions will find the manual just as useful as those aiming for careers in interest such as research, teaching, and industry.

This lab manual is organized flexibly so that chapters and topics may be arranged in almost any order to use in lab activities and writing lab report. Each chapter has been made as self-contained learning outcomes. A list of the goals or objectives is given in the beginning of each exercise. Each chapter related to the experiment has several photographs, tables, and related questions to be studied. Most of the exercise also require some diagrams of specified material and these diagrams should be labeled or be completed by student by the end of lab for the respective chapter.
Most of the chapters are designed in such a manner that students will learn aspect to pursues their career. Current lab manual is designed to be an effective teaching tool at University of Arkansas Rich Mountain.

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Chapter 1: Laboratory Safety and instruments

Objectives:

- To understand the principle of lab safety and regulation
- To define the biohazards chemical and biohazards living cell
- To understand the use of Personal Protective Equipment (PPE).
- To describe the basic importance of the lab instruments

Introduction

Laboratory safety regulations will be strictly enforced. Repeated infractions will result in points lost. All instruments, chemicals and organisms should be handled with proper aseptic technique and universal precautions manner when working in the laboratory. In the interest of safety and accident-prevention, there are general laboratory rules to be followed by all students in laboratory rooms at University of Arkansas Rich Mountain. Faculty and staff members are authorized to deny the use of any laboratory to students who do not adhere to the rules mentioned below.

- Wash your hands with soap and water before entering or leaving the lab.
- Lab experiments have been designed to minimize unnecessary exposure to any hazardous substance. A list of all chemicals, specimens, and/or microbes used in the laboratories is available for your benefit.
- If you have any medical condition or concern, including but not limited to the following: compromised immune system, seizures, epilepsy, severe allergies, or pregnancy, it is your responsibility to consult with your medical care provider regarding any medical issue associated with taking this lab. If you have concerns about a health risk, please communicate accordingly with the Director of Risk Management, Occupational Health & Safety, so that reasonable accommodations can be determined.
- It is the responsibility of the individual using chemicals, operating equipment or manipulating organisms, to know the associated hazards of each and to handle them accordingly. Safety Data Sheets and container labels provide hazard information that must be followed. Materials must be disposed of appropriately per faculty instructions.
- Unauthorized work is prohibited. This includes working after hours without proper authorization and performing procedures that have not been expressly authorized by laboratory staff or faculty. Some areas are designated as off-limits to the students and should not be entered without supervision.
- No food or drink is allowed in the lab.
- Cell phones are not allowed in lab under any circumstances.
- Personal Protective Equipment (PPE); Safety goggles must be worn when working with chemicals. Lab coats/aprons and protective gloves are required when handling chemicals. Footwear impermeable to

liquid spills is recommended. You must limit exposed skin as much as possible, and faculty may enforce more stringent clothing guidelines than those outlined here. If you wear inappropriate clothing to the lab, you will not be allowed to participate. PPE should not be worn outside the lab.

- It is recommended that contact lenses should not be worn in the lab as chemicals can get between the eye and the lens. However, additional PPE or alternative measures can be taken for those required to wear them.
- Be familiar with the location and the use of the following in your laboratory: e.g. broken glass receptacle, bio-hazard waste receptacle, first-aid kit, gas shut-off valves, fire blanket, closest fire alarm, fire extinguisher, eye wash, safety shower, spill kit, telephone, and emergency exits and routes.
- Lab activities have students moving around the lab or involve moving objects. Be alert and aware of what's going on around you at all times.
- Faculty must be notified immediately of any health reaction/issue or accident occurring in the lab. Any spillage or breakage should be cleaned up by the Lab Coordinator, Faculty, or Staff.
- Be organized. Maintain a clean, open work area free of anything except materials directly required for the exercise. Keep laboratory material/equipment away from edges of work surfaces and electrical cords from hanging below the surface of tables or draped across sinks. Use caution when working with electrical equipment to avoid a shock.
- Accidents or injuries must be reported to your instructor as soon as possible. Know the location of the fire blanket, fire extinguisher, shower and eyewash in case of emergency.
- Dispose of wastes as instructed. Hazardous chemicals and microbial cultures must be properly discarded in designated containers and kill areas. We will inform you of the proper disposal procedures. Do not discard any materials that have contacted microbial cultures or hazardous chemicals in sinks or wastebaskets.
- In addition to these rules, faculty may also provide additional safety instructions that are specific to the laboratory course you are taking. Additional safety instructions may be more stringent than the rules outlined here. You must adhere to all safety instructions provided by the laboratory.

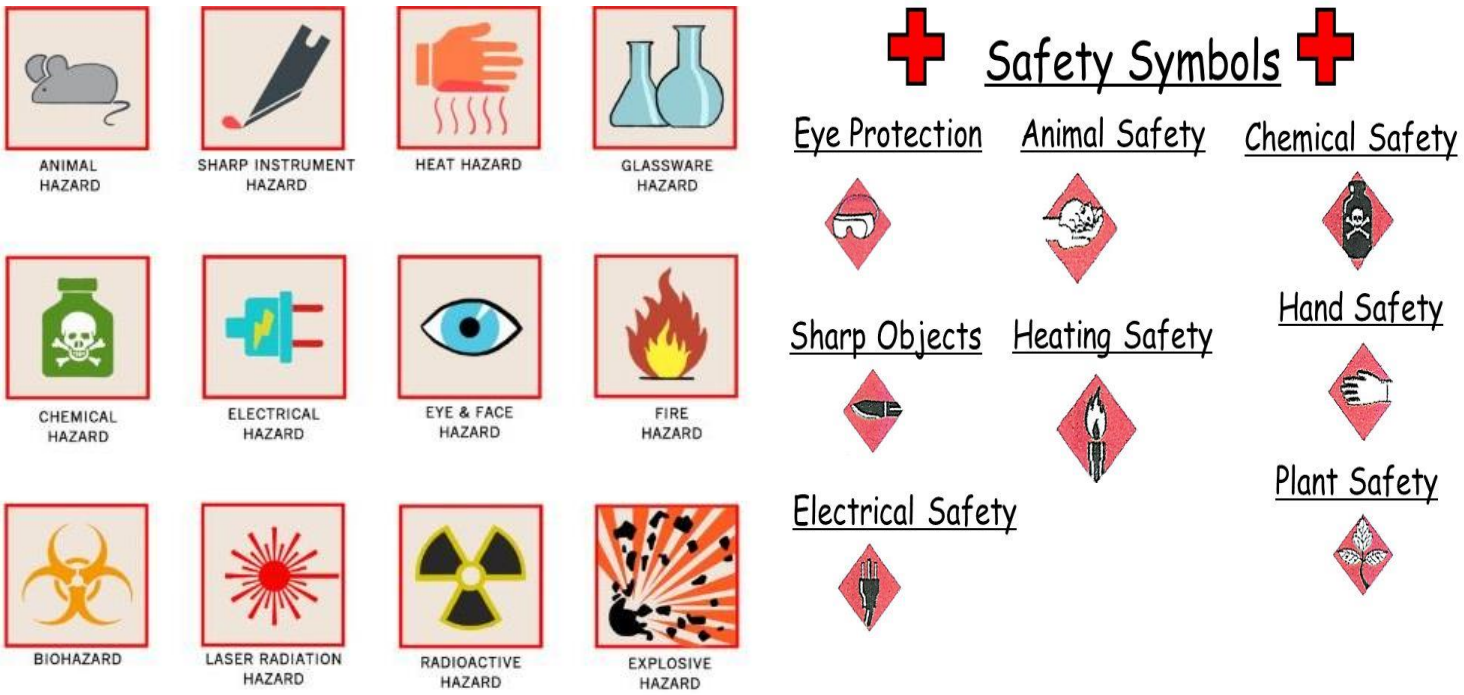


Figure 1.1 Hazards and safety symbols

Lab equipment

As shown in Figure 1.2, the laboratory equipment market is a thriving industry that encompasses a wide range of instruments, devices, and tools used in various scientific and research laboratories. These equipment are designed to facilitate and enhance scientific experiments, measurements, analysis, and other laboratory processes. The demand for laboratory equipment is driven by several factors. Firstly, advancements in scientific research and technology have increased the need for sophisticated and precise instruments to conduct experiments and gather accurate data. Additionally, the growing number of research and testing facilities worldwide, such as academic institutions, pharmaceutical companies, biotechnology firms, and healthcare organizations, has created a significant market for laboratory equipment.

Figure 1.2 Selected laboratory equipment

Laboratory safety regulations will be strictly enforced. Repeated infractions will result in points lost.

All organisms will be considered pathogenic and should be handled with aseptic technique and universal precautions when working in the laboratory.

1. Wash your hands with soap and water before entering or leaving the lab.

2. Wear shoes that completely cover your feet, you cannot wear sandals or other open-toe OR open-heel shoes (clogs are ok).

3. You must wear a lab coat when working in the microbiology lab. You must store your lab coat/shirt in the lab. Your lab coat/shirt may NOT be removed from the room during the semester.

4. Long hair must be tied back in a ponytail, all hats must be removed or turned backward.

5. Accidents or injuries must be reported to your instructor as soon as possible. Know the location **of the fire** blanket, fire extinguisher, shower and eyewash in case of emergency.

6. Do not eat, drink, or chew gum in the lab. Avoid hand-to-mouth activities such as nail biting or chewing on the end of a pencil/pen.

7. Cell phones are not allowed in lab under any circumstances except taking image and lab related purpose.

8. Do not put paper towels used to wipe down the bench in the bench top waste baskets.

9. Bunsen burners should never be left unattended

10. Disinfect your lab space at the start and end of each lab period. To disinfect your area wipe down the table top thoroughly with laboratory disinfectant and a paper towel. When leaving the lab, apply disinfectant to the bench area and leave to air dry (wet).

11. Remove any non-essential materials from your lab workspace, and keep only what you need for conducting the laboratory activity on your bench. Store coats and backpacks where they will not be in the way.

12. At the end of the lab period return all equipment to its place of origin. It is important that we maintain/improve our surroundings for other classes/students.

13. Dispose of wastes as instructed. Hazardous chemicals and microbial cultures must be properly discarded in designated containers and kill areas. We will inform you of the proper disposal procedures. Do not discard any materials that have contacted microbial cultures or hazardous chemicals in sinks or wastebaskets.

Chapter 2: The Scientific Method

Objectives:

- To define science and discuss the characteristics of the science.
- To describe the basic and integrated process skills of science.
- To design and conduct lab experiments.
- To collect data and analysis to compare control and variable group; discuss the influence of independent variable on the dependent variable.
- To discuss scientific method and write scientific theory.

Introduction

The scientific method is the way scientists approach a question. It is an empirical method to the study of biology for acquiring knowledge in systemic order. The scientific method includes careful observation, and applying rigorous skepticism about what is observed, given that cognitive assumptions can distort how one interprets the observation. It involves formulating hypotheses, via induction, based on such observations; the testability of hypotheses, experimental and the measurement-based statistical testing of deductions drawn from the hypotheses; and refinement (or elimination) of the hypotheses based on the experimental findings. These are *principles* of the scientific method, as distinguished from a definitive series of steps applicable to all scientific enterprises The general steps of the scientific method are depicted in the **Figure 2.1** below. The hypothesis, or suggested explanation for the observation, is the basis for setting up experiments. Good experimental design is essential to the scientific method.

The scientific method is central to the study of biology: it is a process of acquiring and verifying information through experimentation. The general steps of the scientific method are depicted in the figure below. The hypothesis, or suggested explanation for the observation, is the basis for setting up experiments. Good experimental design is essential to the scientific method. A few keys to good experimental design include effective use of controls, reproducibility, a large sample size, and multiple trials. In an experiment, in order to determine that any changes that occur are due to investigator manipulation only, there must be some basis for comparison. A control group is necessary to establish this basis of comparison. In the control group, everything is kept the same as the experimental group except for the independent variable. The experimental group is actually being experimented upon. For example, in a drug trial there will be a group that receives the drug (the experimental group) and a group that receives a placebo (the control group). The drug itself is considered the independent variable and any change(s) that occur because of the drug are considered the dependent variable. In order to ensure that it is only the drug causing changes, all other variables must be tightly controlled (such as diet, exercise, smoking, etc.). These are referred to as controlled variables.

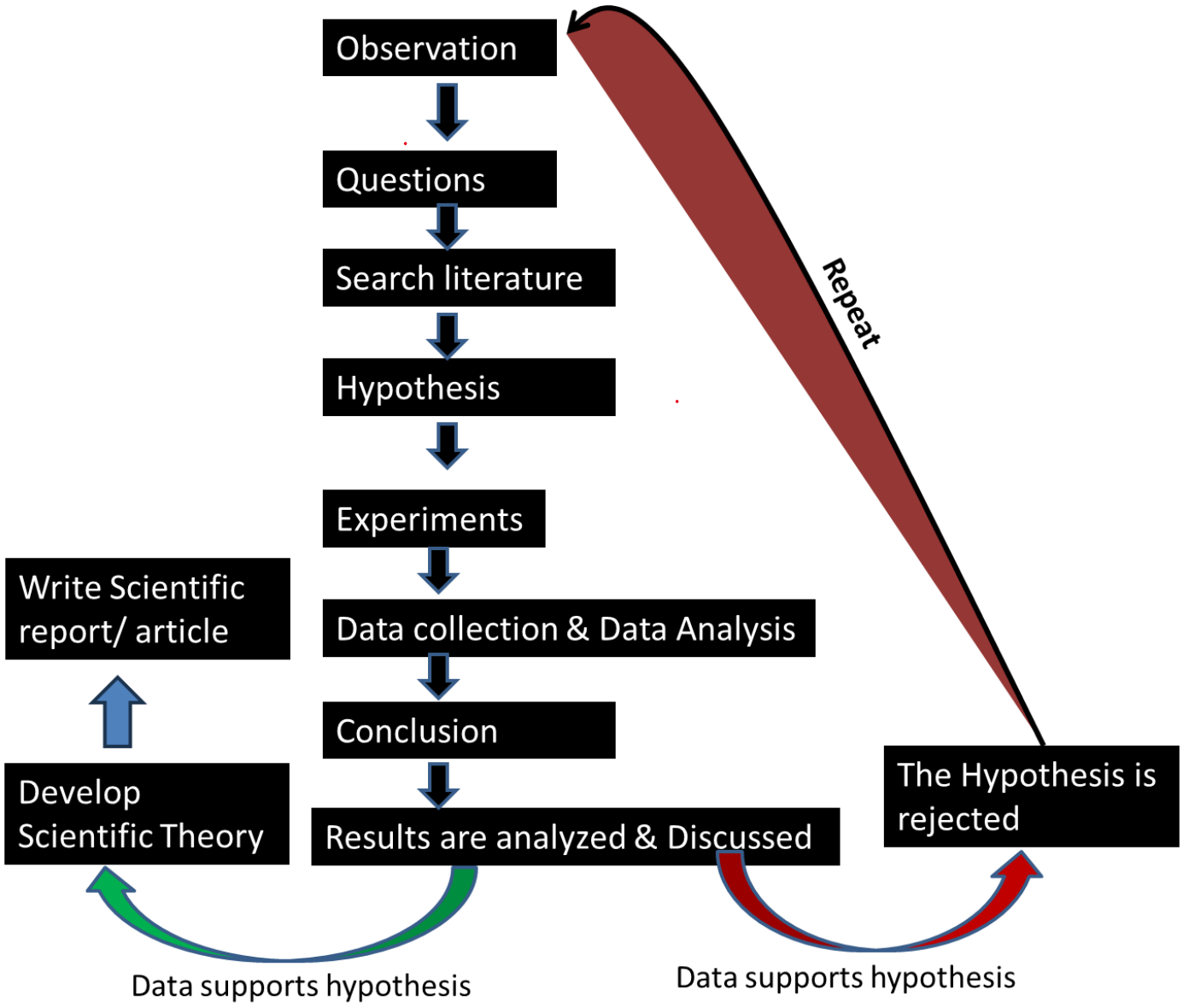


Figure 2.1: Flow char of Scientific method

Experiment on Penicillin Discovery by Sir Alexander Fleming

How Penicillin Was Discovered In 1928, Sir Alexander Fleming was studying Staphylococcus bacteria in growing in culture dishes. He noticed that a mold called Penicillium was also growing in some of the dishes. A clear area existed around the mold because all the bacteria that had grown in this area had died. In the culture dishes without the mold, no clear areas were present. Fleming hypothesized that the mold must be producing a chemical that killed the bacteria. He decided to isolate this substance and test it to see if it would kill bacteria. Fleming transferred the mold to a nutrient broth solution. This solution contained all the materials the mold needed to grow. After the mold grew, he removed it from the nutrient broth. Fleming then added the nutrient broth in which the mold had grown to a culture of bacteria. He observed that the bacteria died which was later used to develop antibiotics used to treat a variety of diseases.

Test yourself.

1. What experiment was conducted by Sir Alexander Fleming in 2028?

2. What was Fleming's hypothesis?

3. How was the hypothesis tested?

4. Do the results indicate that the hypothesis should be rejected?

5. This experiment led to the development of what major medical advancement?
6.

Experiment of The Strange Case of BeriBeri

In 1887 a strange nerve disease attacked the people in the Dutch East Indies. The disease was beriberi. Symptoms of the disease included weakness and loss of appetite, victims often died of heart failure. Scientists thought the disease might be caused by bacteria. They injected chickens with bacteria from the blood of patients with beriberi. The injected chickens became sick. However, so did a group of chickens that were not injected with bacteria. One of the scientists, Dr. Eijkman, noticed something. Before the experiment, all the chickens had eaten whole-grain rice, but during the experiment, the chickens were fed polished rice. Dr. Eijkman researched this interesting case and found that polished rice lacked thiamine, a vitamin necessary for good health.

1. State the Problem.

2. What was the hypothesis?

3. How was the hypothesis tested?

4. Do the results indicate that the hypothesis should be rejected?

5. What should be the new hypothesis and how would you test it?

Hypothesis: Antibiotic B is a better treatment for ulcer than Antibiotic A

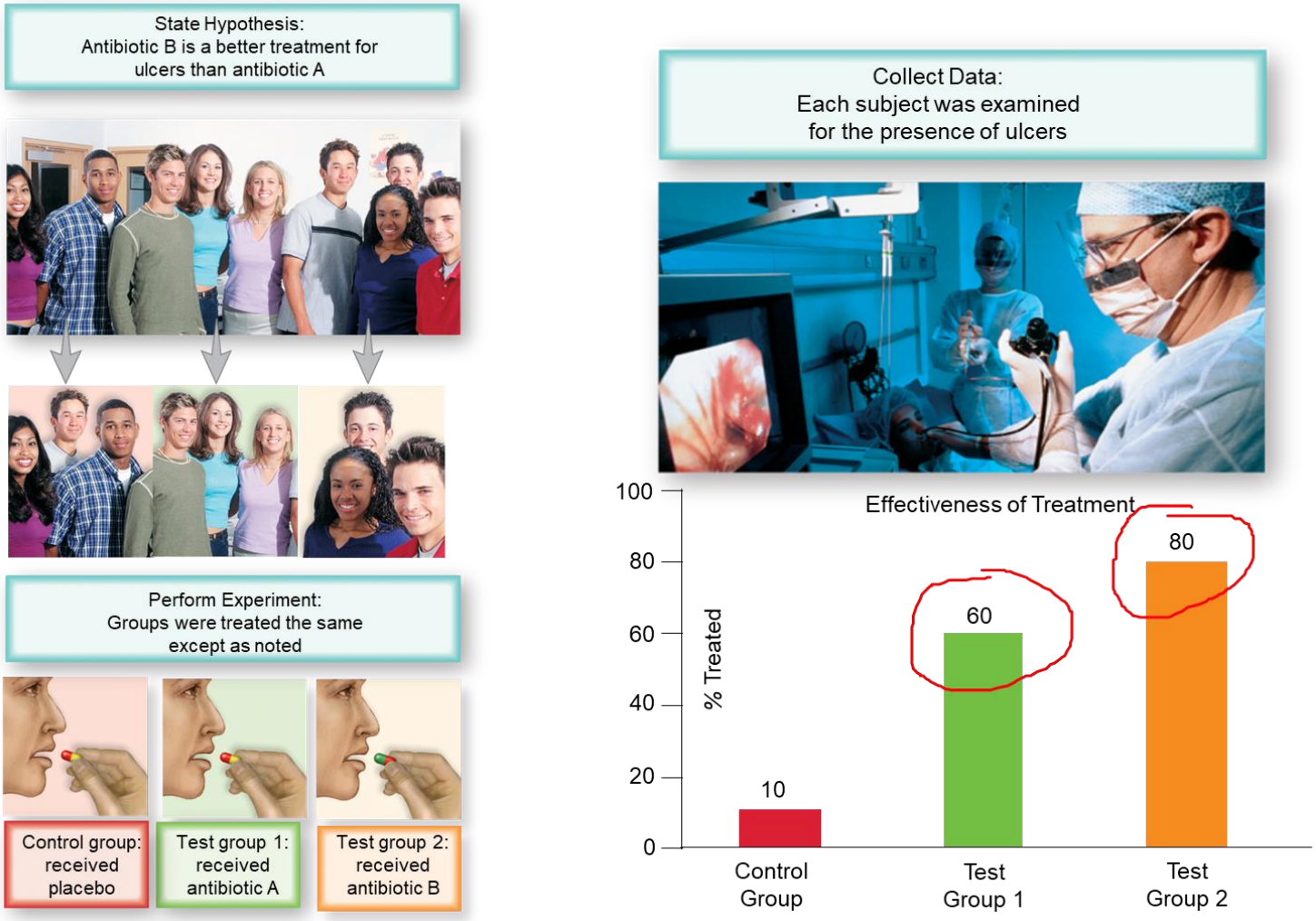


Figure 2.2 Hypothesis states that antibiotic B is better treatment than antibiotic A.

Questions

1. What is basis of the formulation of hypothesis in the ulcer treatment?

2. How was the hypothesis tested?

3. Do the results indicate that the hypothesis should be accepted or rejected? Mention the reason why choose option accepted or rejected.
4. What is difference between Control group and test group?

5. Describe the characteristics of science.

6. What can be done to make better scientific attitude and better education in our nation?

3. Microscopy For Invisible World

Objectives

- To handle and proper care of microscope and dissecting microscope
- To understand the advantage and limitations of both compound light microscope and dissecting microscope
- To utilize the importance of the microscope in microbiology
- To identify and describe the function of the parts of the microscope
- To understand the terminologies like contrast, resolution and determine the total magnification of a microscope using different objectives
- To understand and calculate the field of view at low and high power; and to estimate the size of objects under a microscope.

Introduction

Microscopes are useful tools for visualizing small structures in detail. As we know that many biological organisms and structures such as biological specimens including cells and microorganisms such as bacteria and viruses are difficult or impossible to be seen with the naked eye. Therefore, to observe such specimens they must be magnified to be observed to study their characteristic features like internal structure (**Figure 3.1**).

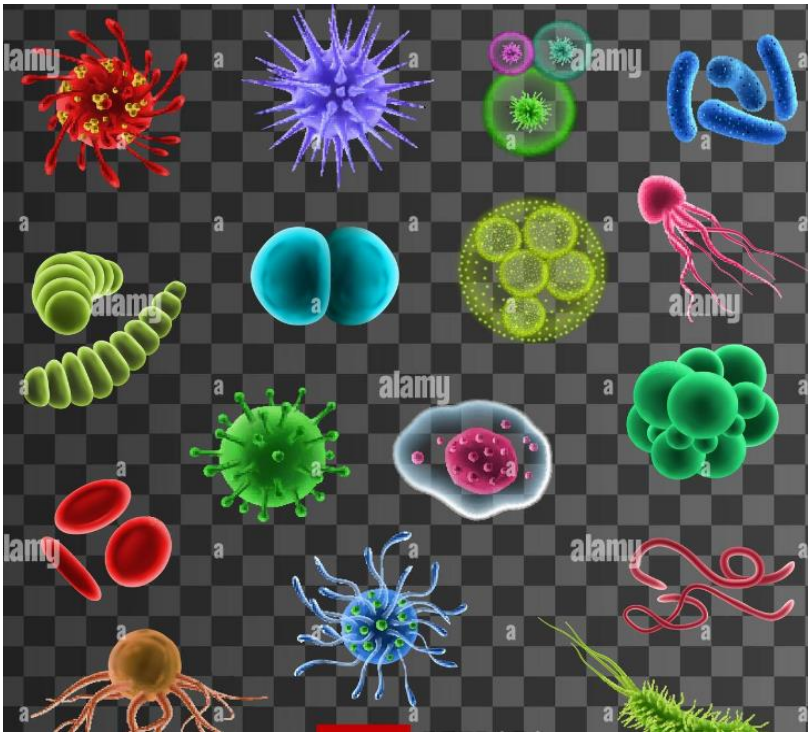


Figure 3.1 False color image of some microorganisms.

In the microbiological science, microscope is the most important and frequently used tools that allows the user to discover the fascinating world of microorganisms. In addition of many biological specimens such as cells and several tissues are difficult or impossible to be seen with the naked eye so they must be magnified to be studied

under microscope **(Figure 3.2)**. Consequently, the basic operation and care of microscopes is an important skill in biology.

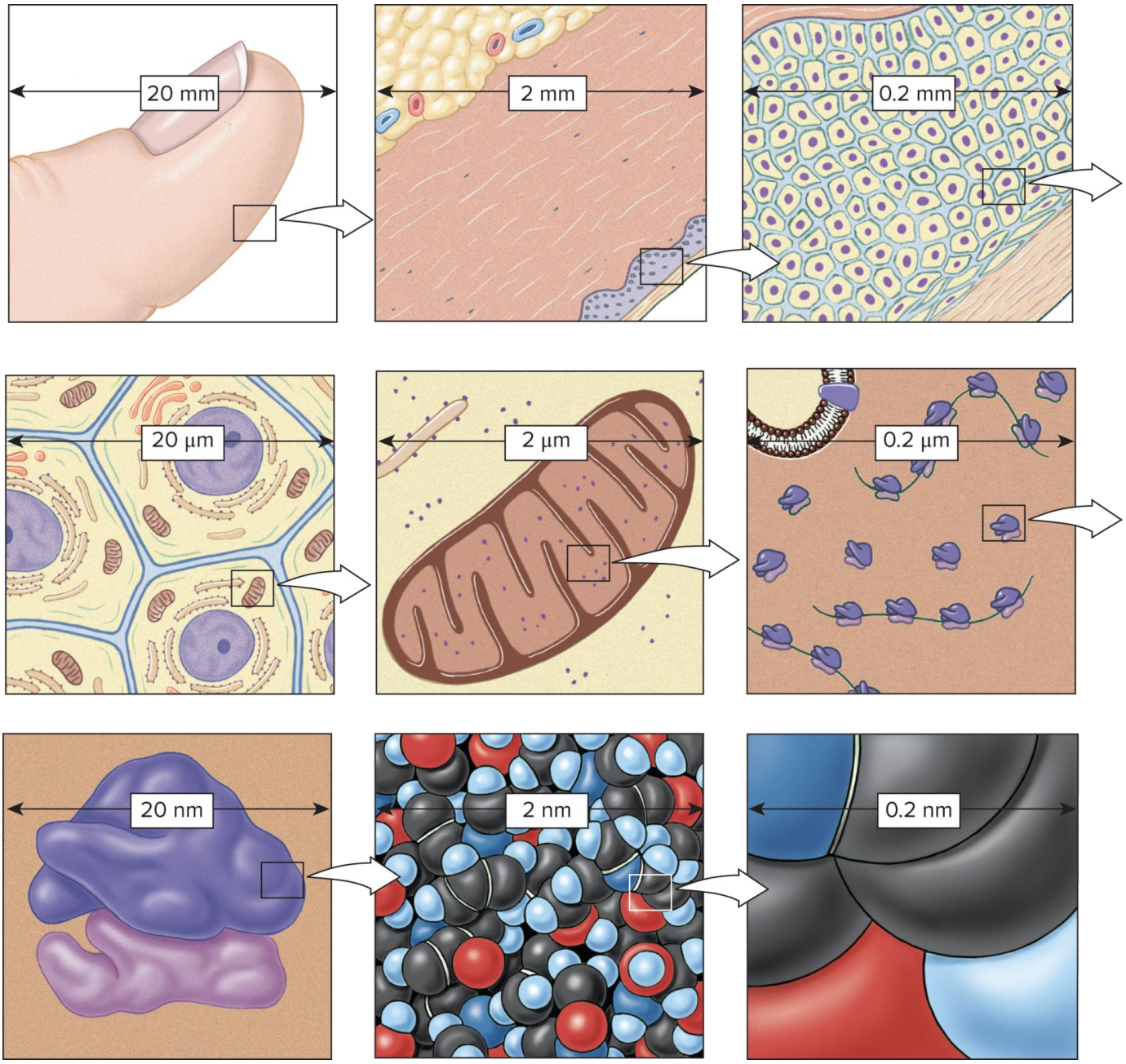


Figure 3.2 Typical human skin cells and their contents. Human skin cells, organelles, and their contents like macromolecules. Diagram shows the diameter of human skin cell is ~ 20 micrometers, mitochondria is ~2 micrometers, ribosome is ~ 20 nanometers, protein molecule is ~2 nanometers and atom is 0.2 nanometers.

Figure 3.3 shows a typical compound light microscope which is frequently uses in the biological and microbiological laboratories. Compound light microscopes pass visible light through two sets of magnifying lenses (the ocular and the objective lenses) to magnify specimens mounted on a glass slide and placed on the flat surface (stage) of the microscope.

There are two frequently used common types of microscopes **(Figure 3. 2)** in the biological and microbiological laboratories. 1. The compound light microscope (commonly known as a light microscope) and, 2. the stereo microscope (commonly known as a dissecting microscope). Compound light microscopes pass visible light through two sets of magnifying lenses (the ocular and the objective lenses) to magnify specimens mounted on a glass slide and placed on the flat surface (stage) of the microscope. The specimens must be sliced very thin in order for light coming from the light source to pass up through them so that they can be viewed. Specimens are sometimes stained with dyes to add contrast and to make structures more easily identifiable. Microscopic examination of cells and tissues allows students to observe the principle of complementarity - how cell and tissue structure determines function.

A light microscope is used to visualize objects flattened onto glass slides in great detail. It typically has a magnification power of up to 40x to 1000x. A light microscope might be used when examining individual cells within living tissue. A dissecting microscope is used to view three-dimensional objects and larger specimens, with a maximum magnification of 100x. This type of microscope might be used to study external features on an object or to examine structures not easily mounted onto flat slides.

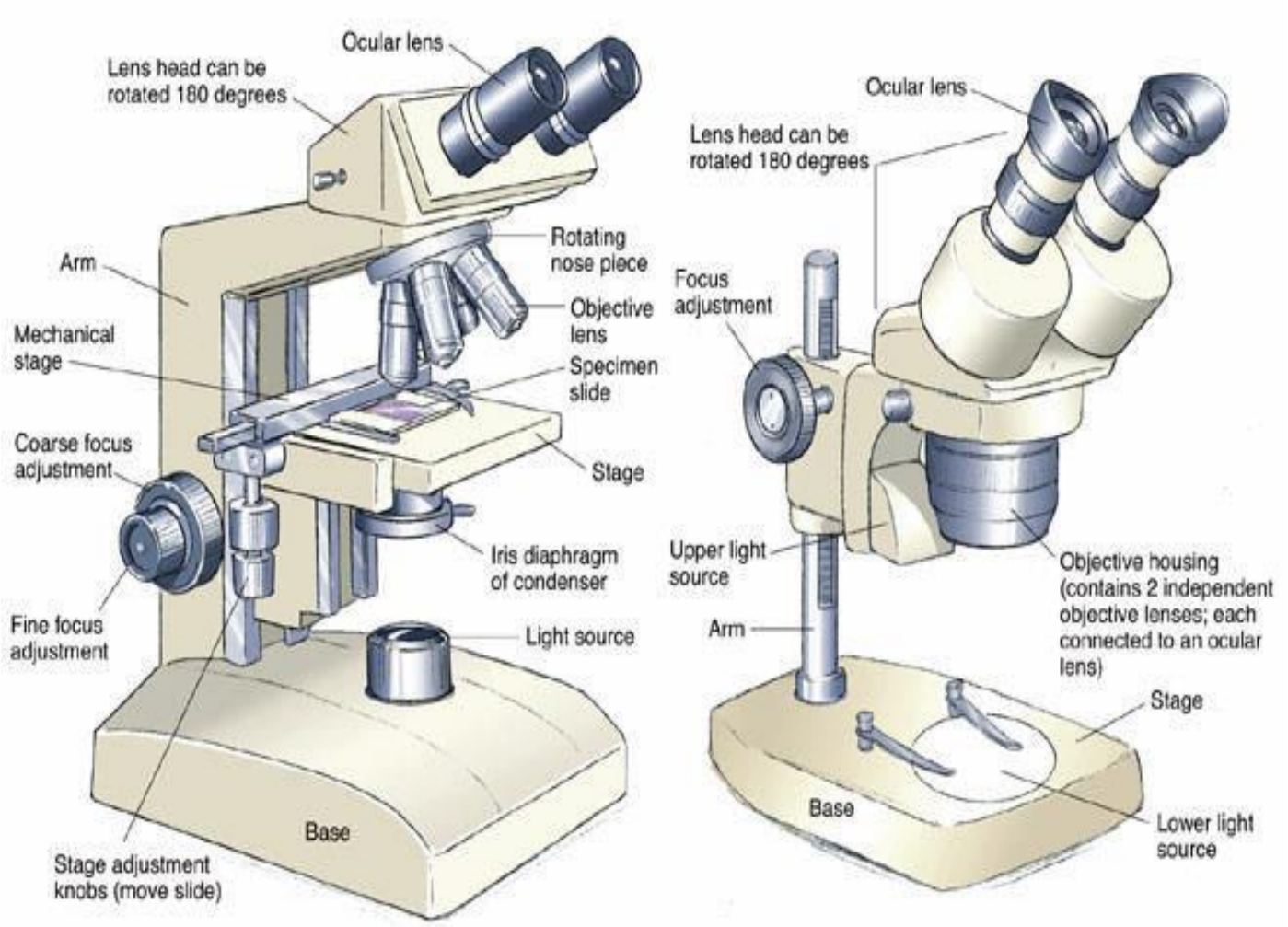
Both microscopes have similar features. Each microscope has an eyepiece as well as a light source. Light microscopes usually have eyepieces that are magnified 10x plus multiple objective lenses that are magnified between 4x and 100x (Figure 3.2 A). The total magnification is calculated by multiplying the eyepiece magnification (10x) by the objective lens magnification (e.g., 40x), for a total magnification would be 400x. On a light microscope, a sample is placed on a glass slide and light is passed through the sample from underneath the stage. This type of microscope shows great detail of small, thin objects. To view thicker objects, the tissue sample must be thinly sliced and prepared on a glass slide in two-dimensions. With a dissecting microscope whole object can be viewed in three dimensions. Samples do not need to be sliced, and larger, live animals can be observed. Light can be passed through from underneath the sample, but also from the top or side using an external light source. However, the magnification power of the dissecting microscope is less powerful than that of a light microscope**.**

Questions

- Write any two major differences between the compound light microscope and the stereo microscope.

- What happens the size of the cells and number of the cells when you go from 4x to 10 x magnification?

- Total magnification of the cell under 100x objective lens magnification_____



A **B**

Figure 3.3 Typical structure and major parts of the compound light microscope (A) & the stereo microscope (B).

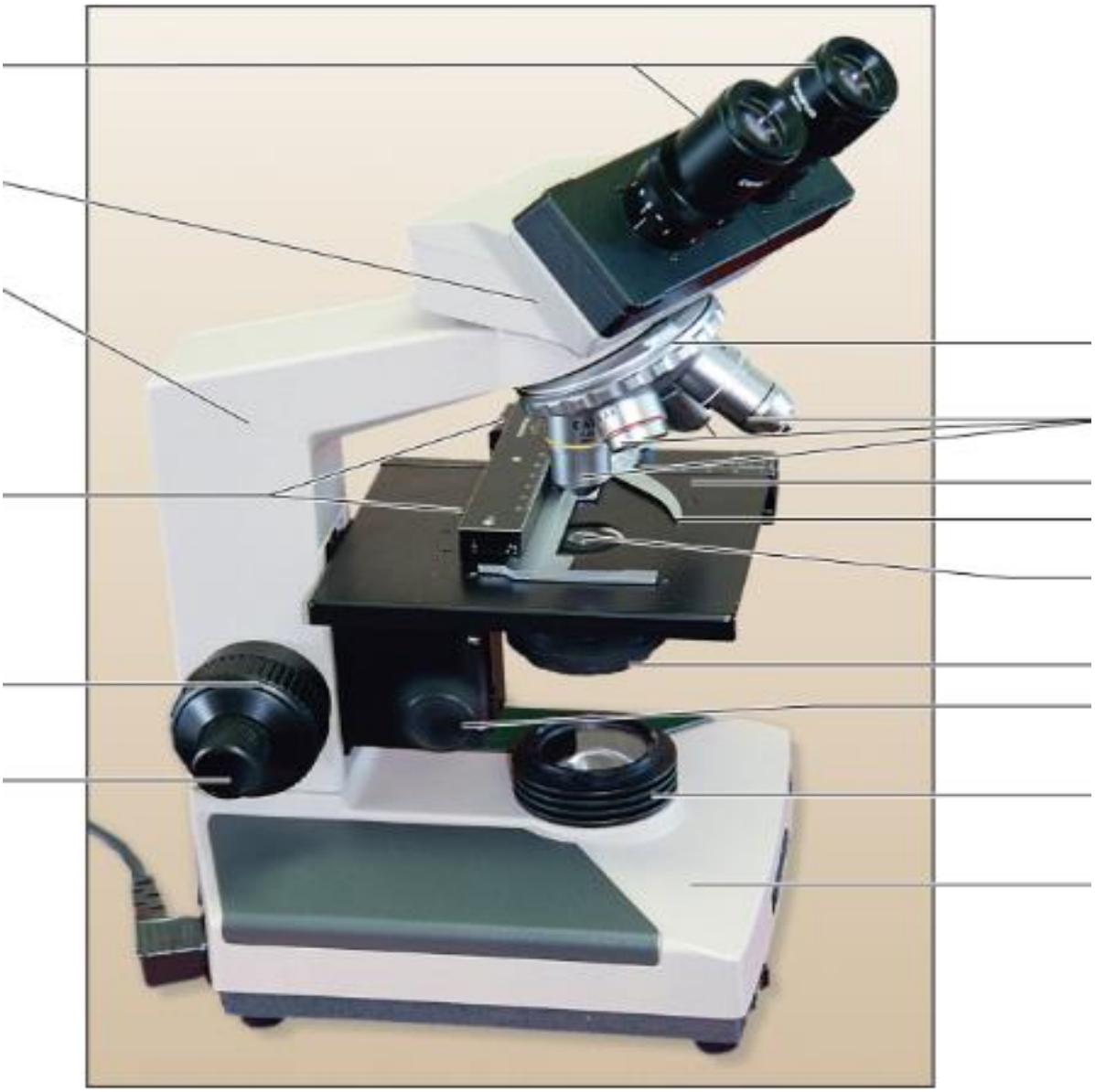


Figure 3.4 Identify and Label the Major Parts of a Compound Light Microscope

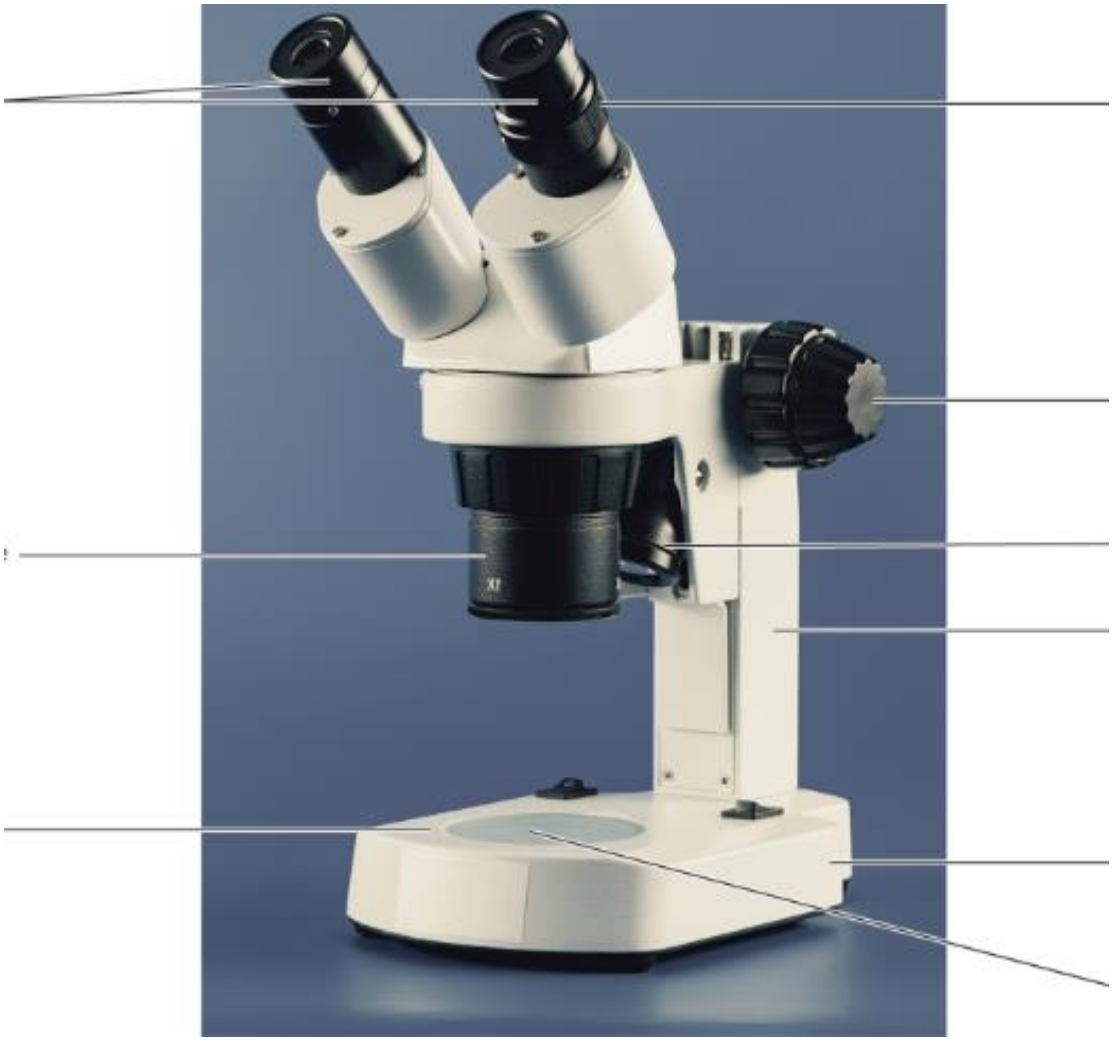


Figure 3.5 Identify and Label the Major Parts of a dissecting (stereoscopic) Microscope

The Dissecting Microscope

The dissecting microscope offer some advantages over a compound microscope. It is extremely easy to operate. Although light microscope can produce high magnification and excellent resolution, it has small distance between the objective lens and specimen. Therefore, it is difficult to manipulate a specimen while observing it with a compound microscope. Specimen that can be observed with compound microscope are limited to those thin enough for light to pass through them. In contrast, a dissecting microscope is used to view objects that are opaque or too large to see with a compound microscope. Most of the dissecting microscopes equipped with 4x to 50x magnification.

Questions

1. What are the advantage of knowing the diameter of the field of view at a given magnification?

2. What are some examples of biological specimens that would be best examined with dissecting microscope instead of a compound microscope?

3. What is the function of each of the following parts of compound and dissecting microscope?
   a. Oculars
   b. Objective
   c. Condenser
   d. Iris diaphragm
   e. Stage
   f. Course adjustment
   g. Fine adjustment

The Compound Microscope

Compound Microscope Parts

A high power or compound microscope achieves higher levels of magnification than a stereo or low power microscope. It is used to view smaller specimens such as cell structures which cannot be seen at lower levels of magnification. Essentially, a compound microscope consists of structural and optical components. However, within these two basic systems, there are some essential components that every microscopist should know and understand. These key microscope parts are illustrated and explained below. This microscope contains a compound set of two lenses, which magnifies the image and allows attaining a total magnification of over 1000X. The ocular lens typically has a magnification of 10X, our lab microscope has four objectives located on a revolving nosepiece: 4X, 10X, 40X and 100X. The 4X objective is also called the ***scanning lens*** because it allows you to scan or search for the specimen you want to observe. This lens provides the broadest ***field of view*** (amount of area observed) because it has the largest aperture in the opening of the lens. As you increase the magnification, the aperture decreases and thus, the field of view also decreases; increasing magnification requires more light or ***illumination*** in order to better observe the specimen. Increasing the magnification of an image requires a higher ***resolution*** that is the ability to distinguish any two separate points. The maximum resolution with this type of microscope is reached at about 1o00X. When calculating total magnification, use the following formula:

Total magnification = power of objective X power of ocular

Circle the objective that produces the smallest field of view:

a. 4X
b. 10X
c. 40X
d. 100X

Table 4.2 Total magnification of Compound Microscope		
Objective lens	**Ocular lens**	**Total Magnification**
4 X (scanning)	**10 X**	
10 X (low power)	**10 X**	
40 X (low power)	**10 X**	
100 X (low power)	**10 X**	

STRUCTURAL COMPONENTS

The three basic, structural components of a compound microscope are the head, base and arm.

- ***Head/Body*** *houses the optical parts in the upper part of the microscope*
- ***Base*** *of the microscope supports the microscope and houses the illuminator*
- ***Arm*** *connects to the base and supports the microscope head. It is also used to carry the microscope.*

When carrying a compound microscope always take care to lift it by both the arm and base, simultaneously.

OPTICAL COMPONENTS

There are two optical systems in a compound microscope: Eyepiece Lenses and Objective Lenses:

Eyepiece or Ocular is what you look through at the top of the microscope. Typically, standard eyepieces have a magnifying power of 10x. Optional eyepieces of varying powers are available, typically from 5x-30x.

Eyepiece Tube holds the eyepieces in place above the objective lens. Binocular microscope heads typically incorporate a diopter adjustment ring that allows for the possible inconsistencies of our eyesight in one or both eyes. The monocular (single eye usage) microscope does not need a diopter. Binocular microscopes also swivel (Interpupillary Adjustment) to allow for different distances between the eyes of different individuals.

Objective Lenses are the primary optical lenses on a microscope. They range from 4x-100x and typically, include, three, four or five on lens on most microscopes. Objectives can be forward or rear-facing.

Nosepiece houses the objectives. The objectives are exposed and are mounted on a rotating turret so that different objectives can be conveniently selected. Standard objectives include 4x, 10x, 40x and 100x although different power objectives are available.

Coarse and Fine Focus knobs are used to focus the microscope. Increasingly, they are coaxial knobs - that is to say they are built on the same axis with the fine focus knob on the outside. Coaxial focus knobs are more convenient since the viewer does not have to grope for a different knob.

Stage is where the specimen to be viewed is placed. A mechanical stage is used when working at higher magnifications where delicate movements of the specimen slide are required.

Stage Clips are used when there is no mechanical stage. The viewer is required to move the slide manually to view different sections of the specimen.

Aperture is the hole in the stage through which the base (transmitted) light reaches the stage.

Illuminator is the light source for a microscope, typically located in the base of the microscope. Most light microscopes use low voltage, halogen bulbs with continuous variable lighting control located within the base.

Condenser is used to collect and focus the light from the illuminator on to the specimen. It is located under the stage often in conjunction with an iris diaphragm.

Iris Diaphragm controls the amount of light reaching the specimen. It is located above the condenser and below the stage. Most high-quality microscopes include an Abbe condenser with an iris diaphragm. Combined, they control both the focus and quantity of light applied to the specimen.

Condenser Focus Knob moves the condenser up or down to control the lighting focus on the specimen

Using A compound Microscope for Lab Activities

Document your observations and draw an image under 40X for prepared slides and by using your phone to snap an image.

Bacteria:

_____

(total magnification: _____)

Cyanobacterial:

_____

(total magnification: _____)

Protist:

_____

(total magnification: _____)

Field of view (FOV)

Field of view for a microscope is the extent of the observable area in distance units. The optics provide a clear and undistorted view in a field around the optical axis, and the field of view is selected from this. The rays that produce the image in this view are generally aberration-free and do not create a significant falloff in image intensity.

Field of view microscope definition in simple terms it is the area you see under the microscope for a particular magnification. Say, for example, you are viewing a cell or specimen under an optical microscope. The diameter of the circle that you see is the field of view of the microscope **(Figure 3.6).** As you increase the magnification, the field of vision is reduced. Depending upon the lens system, this can vary. A crude way of measuring the field of view is by using a ruler under the microscope for a particular magnification. To calculate the field of view of microscope you need to know the eyepiece magnification, field number and objective lens. Once you have this information you can calculate the field of view of the microscope by dividing the field number by the magnification number. Following formula can be used to measure field of view (FOV): **FOV = Field Number (FN*) ÷ Objective Magnification,** *FN* = the Eyepiece F.N.*

Oil Immersion

Microscopic images of biological samples at high magnification are equally beautiful as they are informative, but getting great images is challenging. Are you getting your best possible images? Oil is frequently used with high magnification objectives. You should use an oil immersion lens when you have a specimen that is no thicker than a few tens to one hundred micrometres. Specifically, you should use it when the structures you wish to observe are quite small, perhaps one or two micrometres in dimension or smaller.

When light passes from a material of one refractive index to another (for example: from glass to air), it bends (**Figure 3.8)** . In the space between the microscope objective lens and the slide (where air is), light is refracted, the light scatters and it is lost. The refractive index of air is approximately 1.0, while the refractive index of glass is approximately 1.5. When light passes through both glass and air it is refracted. Light of different wavelengths bends at different angles, so as objects are magnified more, images become less distinct.

Basically, when using lower magnification microscope objective lenses (4x, 10x, 40x) the light refraction is not usually noticeable. However, once you use the 100x objective lens, the light refraction when using a dry lens is noticeable. If you can reduce the amount of light refraction, more light passing through the microscope slide will be directed through the very narrow diameter of a higher power objective lens. In microscopy, more light = clear and crisp images. By placing a substance such as immersion oil with a refractive index equal to that of the glass slide in the space filled with air, more light is directed through the

objective and a clearer image is observed.

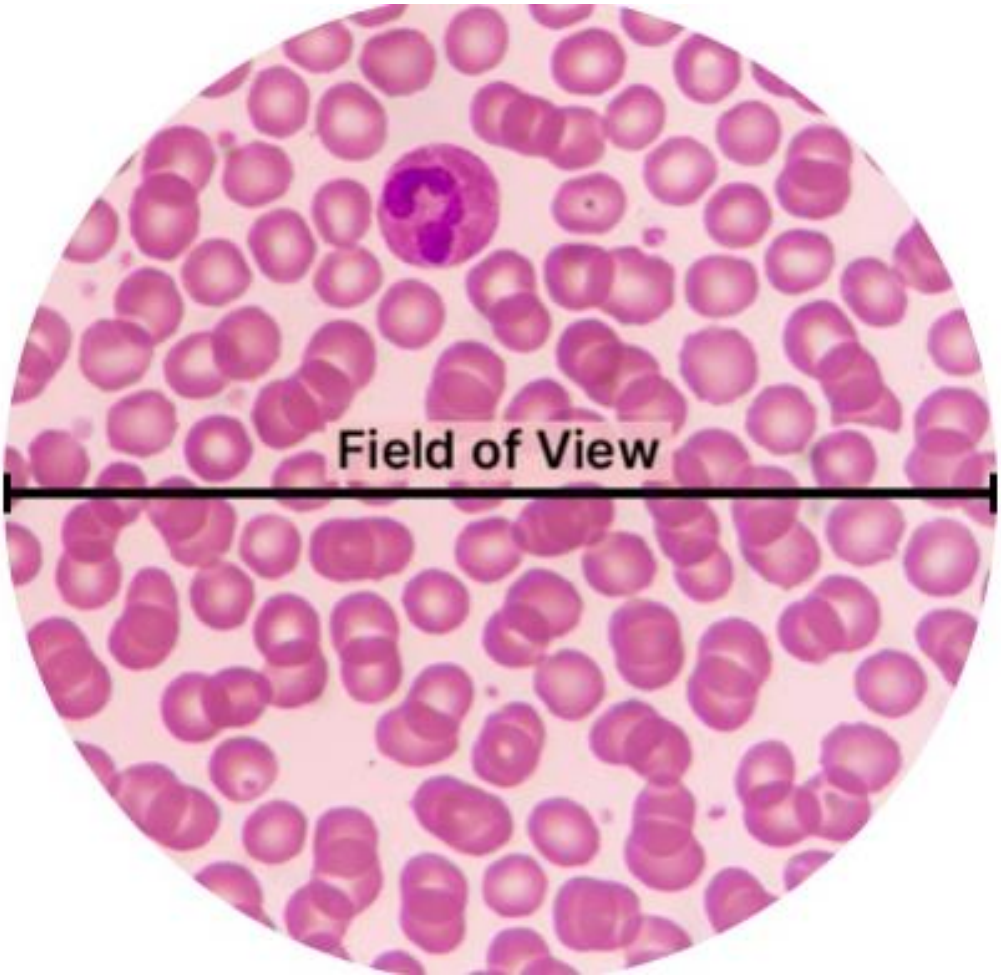


Figure 3.6 The diameter of the circle that you see is the field of view of the microscope.

As you increase the magnification, the field of vision is reduced. Depending upon the lens system, this can vary. A crude way of measuring the field of view is by using a ruler under the microscope for a particular magnification. When you switch eyepieces or objective lenses, ensure to repeat the FOV calculations with the new inputs on field number and magnifications. For objects that require higher magnifications, convert your measurements from millimeters to micrometers. Higher power lenses will allow you to view tiny objects, so the angle of view will be small; low power lenses will do the opposite and let you view bigger (wider) objects.

Take a look at the images captured below. The images were captured using the compound microscope with the 100x Achromat objective lens and the 100x Plan Achromat objective lens. Observation was performed dry and with immersion oil. Notice the difference in image quality between the images captured dry versus those captured with immersion oil. For example, oil immersion dramatically enhances the visualization of specimen like duodenum image under 100X with (Figure 3.7 A) and without (Figure 3.7 B) immersion oil.

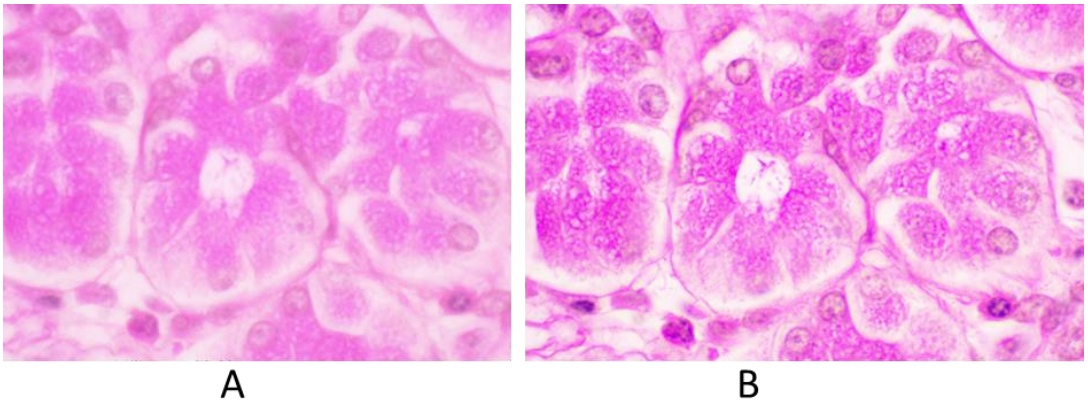


Figure 3.7 Microscopy image of duodenum captured using Plan Achromat 100x objective lens without immersion oil (A) and with immersion oil (B)

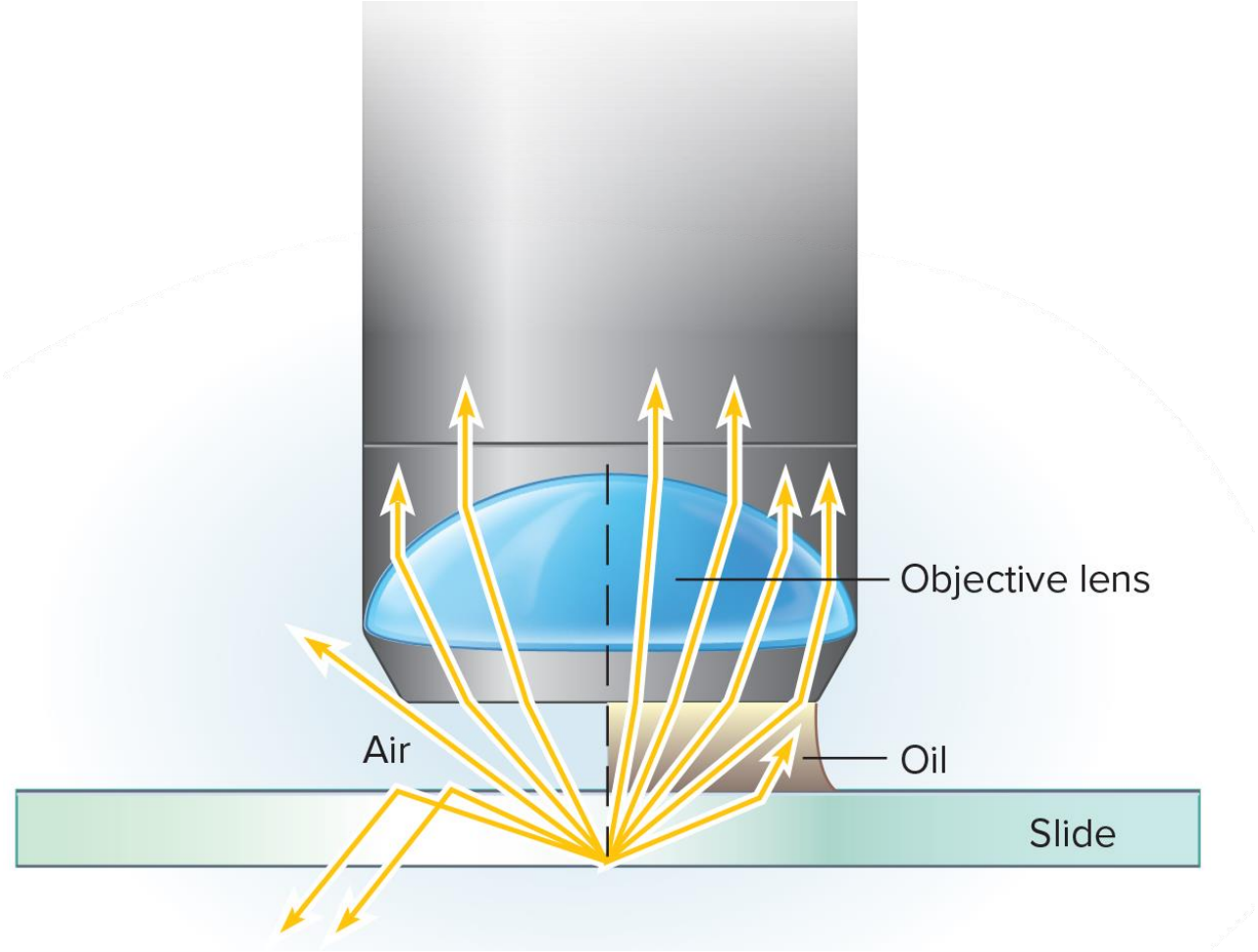


Figure 3.8 Working scheme of the an oil immersion lens

How to Use Microscope Immersion Oil

Before using immersion oil, make sure that your 100x objective lens is made for use with immersion oil.

1. Begin by focusing your sample using the 40x objective lens. Rotate the objective lens part way between the 40x and 100x lens so you can reach the cover slip on your slide.
2. Place a drop of immersion oil on the top of your cover slip and another drop directly on your 100x oil objective lens.
3. Slowly rotate your 100x oil objective lens into place and adjust the fine focus until you get a crisp and clear image.
4. When finished viewing with your 100x oil immersion lens, carefully wipe the oil from all glass surfaces using a piece of lens cleaning paper.
5. Use a second piece of lens paper moistened with a small amount of alcohol (ethyl or isopropyl) or lens cleaning solution, wiping all glass surfaces again to remove any remaining oil. Failing to remove immersion oil from lenses will result in hardened oil on lenses that will affect future clarity.
6. If you need to remove immersion oil that has been left on a lens and hardened, moisten lens paper with a small amount of xylene or microscope lens cleaning solution. Gently wipe lens surfaces, allowing enough time for the solution to soften the hardened oil. Once oil is removed wipe surfaces again.

When To Use Microscope Immersion Oil?

- Immersion oil should only be used if you have an immersion oil lens. The lens will actually have "oil", "immersion" or "HI" (homogeneous immersion) printed on it.
- Immersion oil is best for viewing samples that are dead or are not moving and no thicker than a few micrometers. Very small moving protists can occasionally be viewed with immersion oil, but the best specimens for oil immersion do not move.
- Oil immersion is required when viewing individual bacteria strands or details of striations in skeletal muscle.
- Immersion oil should be used anytime you want to view a clearer image at 1000x.
- There are several disadvantages to using immersion oil. For example, a wet mount slide must be incredibly secure in order to use immersion oil with it. If switching between a dry 40x lens and a 100x oil lens, care must be taken not to get oil on the 40x lens, which could damage it.

Examine and sketch the given specimen under microscope with and without immersion oil. Also take a snap using your mobile for both conditions.

Without Immersion Oil

Specimen_____

Magnification_____

Without Immersion Oil

Specimen_____

Magnification_____

Specimen_____

Magnification_____

Specimen_____

Magnification_____

1. Preparing a Wet Mount:

Materials

- Compound Microscope
- Blank clean slide
- Kimwives
- Pondwater or any other specimens like onion cell or *Elodia* leaf

Procedure

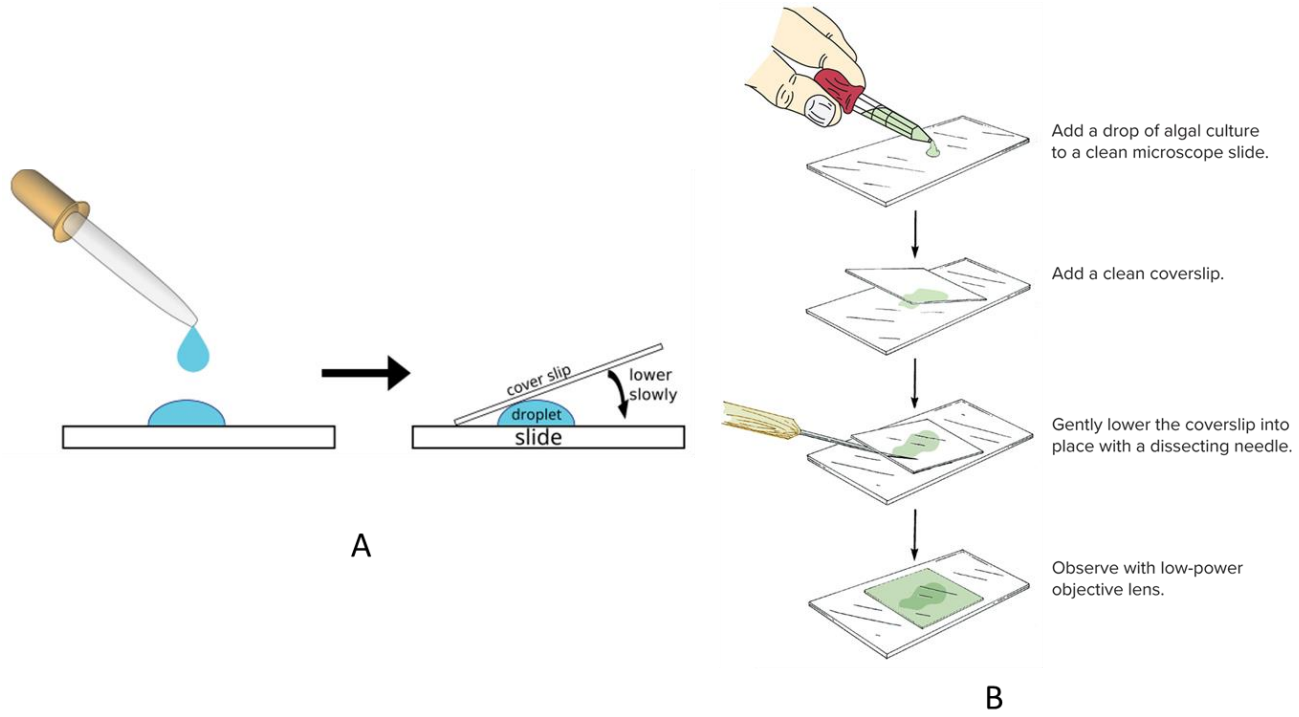


Figure 3.9 Preparing and viewing a wet mount of pound water

Viewing prepared slides

1. Obtain a microscope. Always carry the microscope upright, chest high and with both hands, one hand holding on to the arm and the other under the base. Place the microscope onto the laboratory bench. If you need to reposition the microscope, lift the microscope carefully and reposition the microscope. **Do not drag your microscope across the laboratory bench.** Dragging the microscope can damage the lenses and illuminator. Keep your microscope away from the edge of the desk. Do not rest it on books or papers. Adjust your lab stool or chair for the proper viewing height.

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2. Plug in the microscope and turn on the light. You should see light coming through the condenser and through the stage. If your microscope light does not turn on, make sure the cord/plug on the back is securely plugged in and the GFI (ground fault interrupter) trip on the power outlet is on.
3. Obtain a prepared slide from the slide tray in the common materials area.
4. Open the mechanical stage arm and slide the slide into place. Release the stage arm. The coverslip should be "up" or closest to the objective. If the slide is upside down, you will not see the bacteria under oil immersion.
5. Use the mechanical stage control knobs to position the specimen over the light passing through the stage.
6. Use the iris diaphragm slider to adjust the light. Start with your condenser diaphragm half open. As you increase magnification, you wi need to increase the amount of light passing through your specimen. Viewing specimens at higher magnification requires more light.
7. Grab the nosepiece and rotate the 4× objective over the specimen. Always start focusing with the scanning objective. Do not move to the next higher objective if you do not have a sharp image at the current magnification. If you do not see the object at low power, you won't see it at high power either.
8. Grasp the coarse adjustment knobs and rotate the knobs to raise the stage to its highest point.
9. Adjust the oculars for your interpupillary distance.
10. Look into the oculars and use the coarse adjustment knob to slowly lower the stage. The slide will slowly come into focus. Look for colored, dustlike spots in your **field of view.** Use the fine adjustment knobs to sharpen the image.
11. Use the mechanical stage adjustment knobs to move the dustlike spots into the center of your field of view. If your image is not centered, it may not be in your field of view when you rotate to the next objective.
12. Rotate the 10× objective over the slide. Because your microscopes are **parfocal,** your specimen should still be visible and in relatively
13. Rotate the 40× or 45× objective over the slide. Use the fine adjustment knob to sharpen your image. You should not have to use the coarse adjustment knob. Center your specimen in the center of your field of view.
14. To increase the available **working distance,** rotate the 4× objective over the slide. **DO NOT MOVE THE STAGE** or **THE MECHANICAL STAGE.** Add a drop of immersion oil directly to the slide.
15. Rotate the oil immersion lens into position over the slide. The objective should enter the oil. **Do not** move the stage down; the lens must be in the oil and very close to the slide. If you follow the correct focusing procedure, you will not break the slide.
16. Use the fine adjustment knob to sharpen your image. The **depth of field** is not very large at this magnification. As you perform fine focus adjustments, focusing up and down, individual cells will come in and out of focus quickly.

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17. Record your results in your lab report.
18. Use this same procedure to observe all of the prepared slides and record your observations of these slides in your lab report.
19. All prepared slides must be cleaned (wipe with paper towel and lens cleaner) and returned to the correct slide tray.

1. Obtain a clean blank slide and coverslip.
2. Acquire a pipette or eyedropper.
3. Gently squeeze the bulb of the pipette and insert it into the container of pond water. Place the pipette near any solid material or noticeably chunky material in the sample. Release the bulb.
4. Squeeze the bulb gently to dispense **one** drop of pond water onto the clean slide. Too much liquid will make observing the specimen more difficult.
5. Place the plastic coverslip over the drop.
6. View the slide (see steps 4–13 of "Viewing prepared slides"). You should not use oil immersion with this specimen.
7. Draw in your lab report two different types of organisms from your pond water sample.
8. When you have completed your drawings, dispose of your slide and coverslip appropriately.

4X 10X 40X

Type 1

4X 10X 40X

Type 2

Chapter 4: Aseptic Techniques

Objectives

- To perform appropriate hand hygiene and Prevent contamination of the specific microorganism we are working with.
- To use standard precautions and use category-specific, transmission-based precautions.
- Maintain a sterile field and equipment.
- To Prevent contamination of the room and personnel with the microorganism we are working with
- Apply and safely remove sterile gloves and personal protective equipment; and dispose of contaminated wastes appropriately.

Introduction

In microbiology lab, you will be working with several pathogenic species of bacteria. To effectively study individual bacteria, it must be done so in the absence of other contaminating bacteria. In order to maintain species in pure culture, aseptic techniques must be followed. Appropriate personal hygiene is the start to an effective aseptic technique. Remember that bacteria are in the air as well as on the skin, the counter, and all objects and equipment that have not been sterilized. The most important tool for transferring cultures is the wire inoculating needle or loop. It can be quickly sterilized by heating it to red hot in a Bunsen burner flame (**Figure 4.1)**. Adjust the air inlets of the burner so that there are a hotter inner cone and the cooler outer flame. A dry needle may be sterilized by holding it at a 30-degree angle in the outer part of the flame. A wet loop with bacteria on it should first be held in the inner part of the flame to avoid spattering, and then heated until red hot in the outer part of the flame. Always flame the loop immediately before and after use! Allow it to cool before picking up an inoculum of bacteria (or you will kill the bacteria). Hold the loop or wire handle like a pencil.

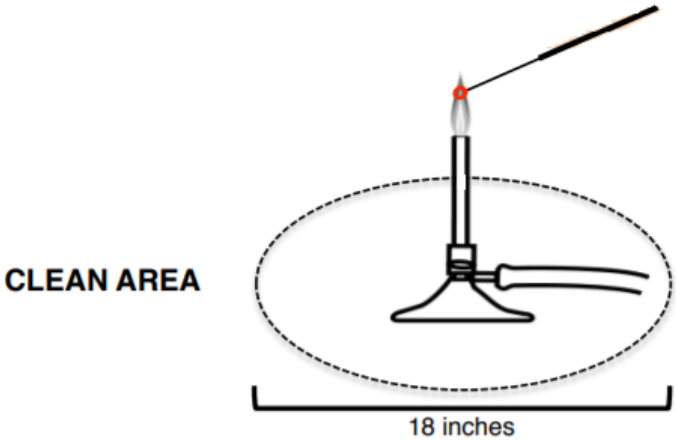


Figure 4.1: Bunsen burner flame

The human skin inhabits multiple, naturally occurring, bacterial populations. Any contamination in the growth medium may potentially ruin the results of the experiment. In this experiment, four different aseptic techniques will be carried out: Autoclaving, moist heat, filtration, and UV light. Autoclaving is an extremely effective method of sterilization for items that are not heat sensitive.Autoclaving will kill all

spores by subjecting each item to 121 degrees Celsius at 15psi for 15 minutes. Moist heat sterilization subjects bacteria to a boiling water bath to kill bacteria. Filtration removes bacteria from a liquid culture, and it's used to sterilize heat sensitive liquids. Bacteria can also be killed by exposure to UV rays. The exposure of radiation damages the DNA of bacteria by causing thymine dimers which cause mutations. Each technique is appropriate for a certain type of bacteria. The bacteria will be observed after each technique is complete and the

sterilization ability will be compared.

Remember to:

1. Always stand or carry tubes in a rack.
2. Not lay the cap down or touch anything with it.
3. Not remove the lid unnecessarily or for prolonged periods of time.
4. Always put the lid of plates face down.

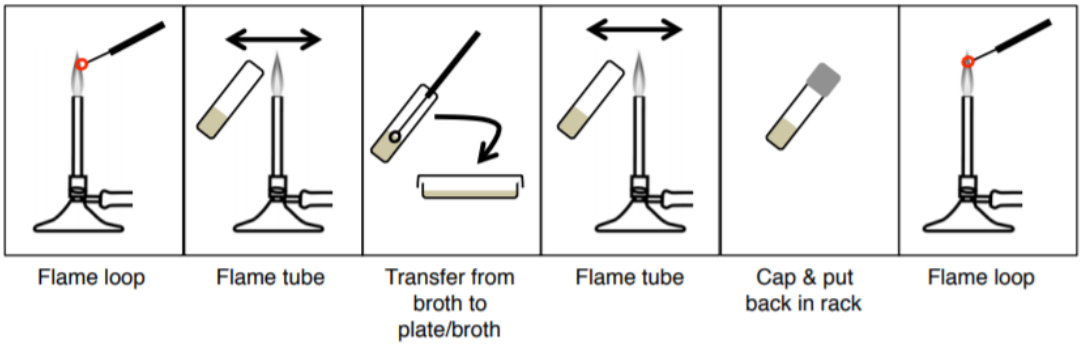


Figure 4.2 Bunsen burner being used to sterilize incubating instrument and transfer bacteria from stock media to culture media.

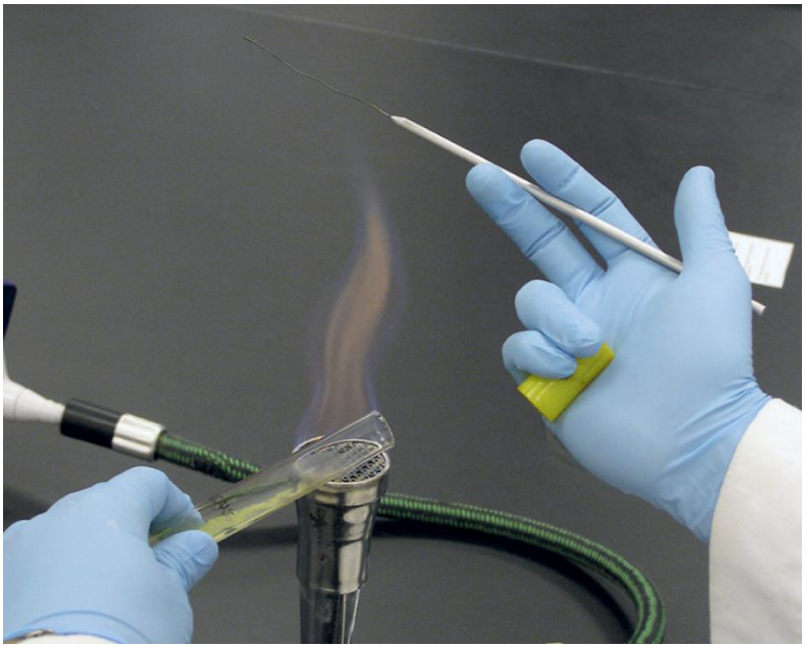


Figure 4. 3. Bunsen burner being used to sterilize incubating instrument, Opening the tube, cap and loop

Effect of Moist Heat and Autoclaving

Procedure

1. Obtain 4 tubes. 3 of the tubes contain spore strips (*Bacillus stearothermophilus or any other bacteria*).
2. One of the tubes with a strip will be your control and will receive no heat treatment. The fourth sterile tube (with no spore strip) will act as a control of your aseptic pipetting technique.
3. Set one tube aside for autoclaving. Autoclave for 15 minutes.
4. The second tube should be placed into a boiling water bath for 15 minutes.
5. After heating treatments, add 2 ml of Tryptic Soy broth to each of the 4 tubes.
6. Incubate at 56ºC until the next lab period.
7. Record your results as presence/absence (+/-) of growth.

Tube	Results	Remarks
Control with spore strip	+	Growth expected, nothing was present to inhibit growth
Control without spore strip	-	No growth expected, no spores were present
Autoclave with spore strip	-	Totally effective in killing all bacteria spores
Boiling with spore strip	+	Does not completely sterilize. Not as effective as autoclave.

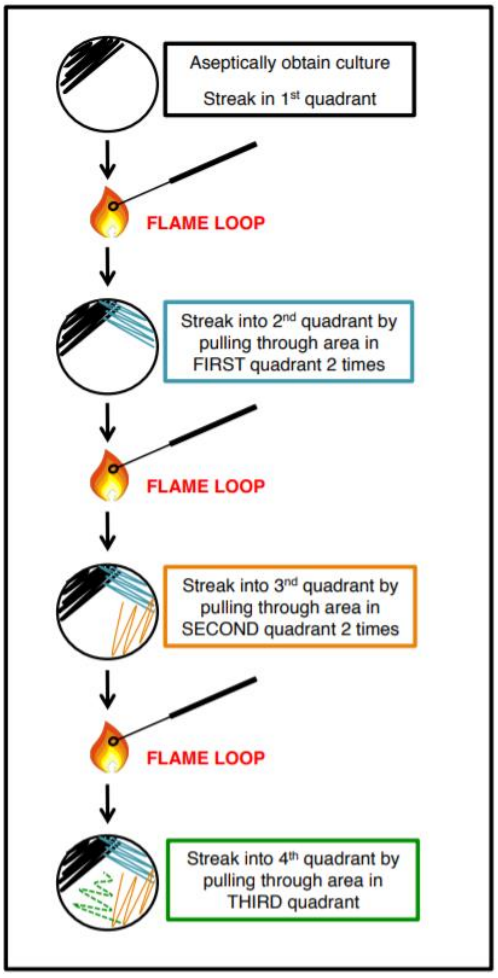


Figure 4. 4 How To Streak For Isolated Colonies (SFIC)

Mixed cultures (more than one species) can be isolated using the streak plate technique. The goal is to acquire a pure culture of one species of bacteria, in a single colony, from a mixed culture We do this by separating the microbes on the surface of agar with quadrant streaking, this method DILUTES the bacteria. The goal is not to have bacteria in all four quadrants but rather to get single isolated colonies.

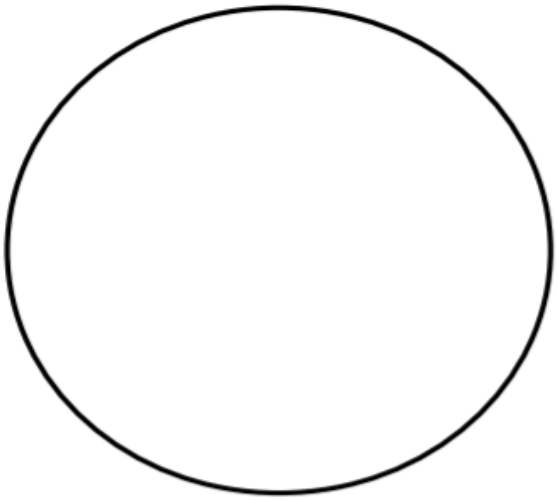


Figure 4.5: Streak plate technique to prepare isolated colony. Steak For isolated colonies (SFIC)

Typed of growth media

SLANT: solid medium made with agar and various nutrients and indicators. Slanting gives the bacteria a greater surface area on which to grow in a tube. Agar slants are also useful in maintaining bacterial cultures, more so than stacks of Petri dishes. Multiple cultures are easily placed into test tube racks and stored under refrigeration. Bacteria are inoculated onto a slant using a loop and grow in the surface of the agar.

SLANT/DEEP: solid medium made with agar and various nutrients and indicators. Similar to a slant but creates a deep zone, commonly called the 'butt'. This type of culture medium gives the ability to grow bacteria in both an aerobic, oxygen-rich, environment (surface of the slant) and an anaerobic, oxygen

deficient, environment (butt of slant). Slant/deeps are inoculated by stabbing a needle into the butt and then immediately streaking across the surface of the slant.

DEEP: solid medium made with agar and various nutrients and indicators. This type of culture is used for the growth of anaerobic bacteria which grow in the absence of oxygen and are inoculated by stabbing the media with a needle.

BROTH: liquid medium made with various nutrients and indicators. Allows for the growth of large volumes of bacteria, the level of growth can be assessed based on the turbidity (cloudiness) of the culture. Bacteria are inoculated into a broth using a loop.

BROTH+DURHAM TUBE: liquid medium made with various nutrients and indicators in which an upside-down smaller tube, called a Durham tube, is placed. Durham tubes are used to detect the production of gases, such as CO2 or N2, by microorganisms. The tube is initially filled with the medium and then collects gas as the bacteria grow, creating a bubble. Bacteria are inoculated into a broth+Durham tube using a loop.

PLATE: solid medium made with agar and various nutrients and indicators. Can be made in Petri dishes of various sizes. Plates are particularly helpful in isolating a specific species of bacteria, which is not possible in a liquid medium. Using the SFIC technique bacteria can be diluted until individual colonies are formed. Bacteria are inoculated onto a plate using a loop

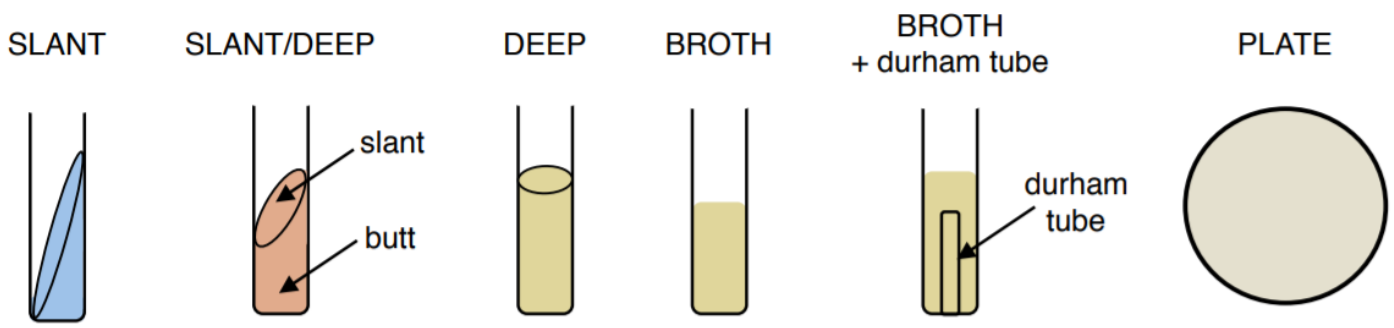


Figure 4.6: Different types of media for bacterial growth

Note: *Please be aware that a loop will collect much higher concentrations of bacteria from a plate than from a broth. Therefore, when using the SFIC technique please consider what media/medium you are taking the bacteria from.*

Aseptic Transfer Of Bacteria:

1. You will be provided with Staphylococus epidermidis in a broth and on a plate. These will be shared with the table and will be found at the front of each table in a white plastic rack.

2. Label 2 broth tubes, 2 slant tubes, and 2 plates.

3. Aseptically transfer bacteria from your BROTH culture to a broth, a slant, and a plate using your loop.

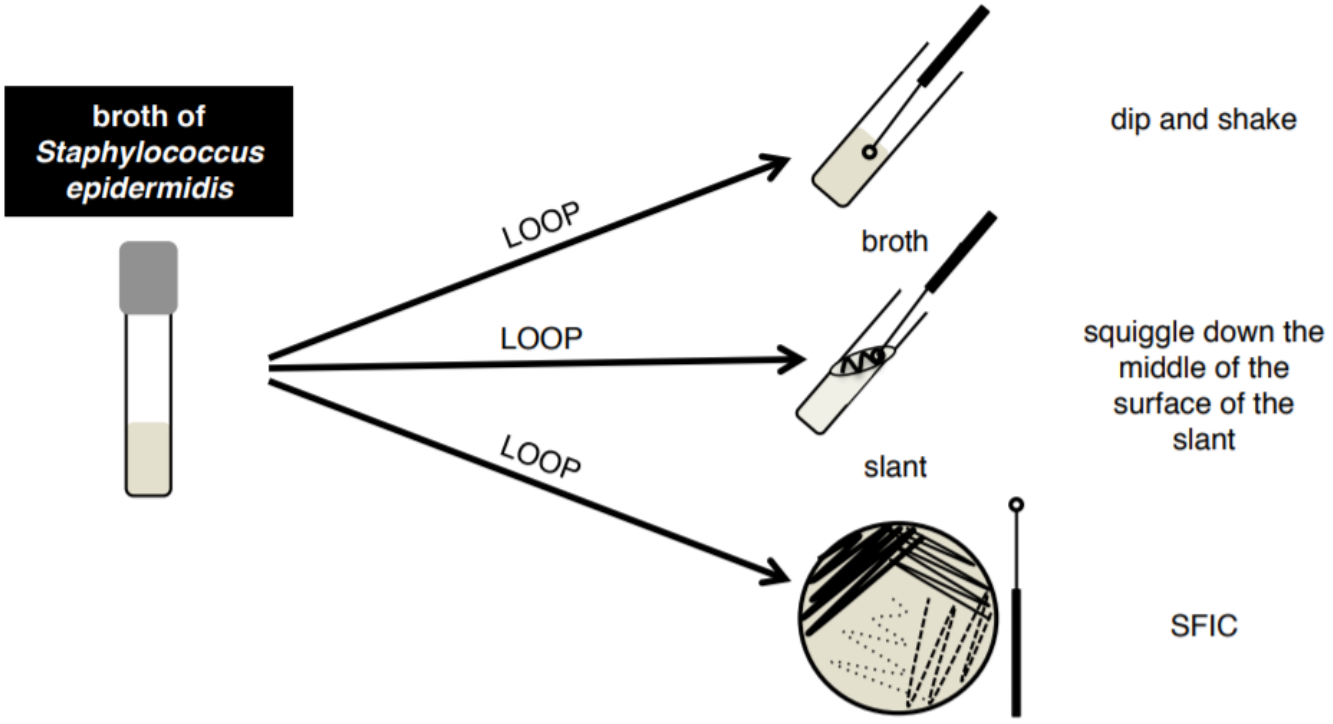


Figure 4.7: Transfer bacteria from broth culture to a broth, a slant and on a plate

Result and Discussion: Check your culture and interpret your data as result and discussion

As can be seen from the case file given at the beginning of this activity, the correct application of aseptic technique is important not only in the laboratory but also in the hospital or clinical environment. Bacteria are everywhere and on everything. Proper usage of aseptic technique will not only prevent contamination of your experimental media but could also have prevented the death of the patient in this case file.

Start here:

Bacterial Shape and Arrangement:

Bacteria are described by three basic criteria: size, shape, and arrangement. The units used to measure organisms seen under a microscope are micrometers (µm). A micrometer is one-millionth of a meter. Most microbes are around 1 µm in size. Viruses are typically 1/10 that size. Animal cells are typically around 10 µm in size. The three most common shapes are the rod (bacillus), the sphere (coccus), and the spiral type (vibrio).

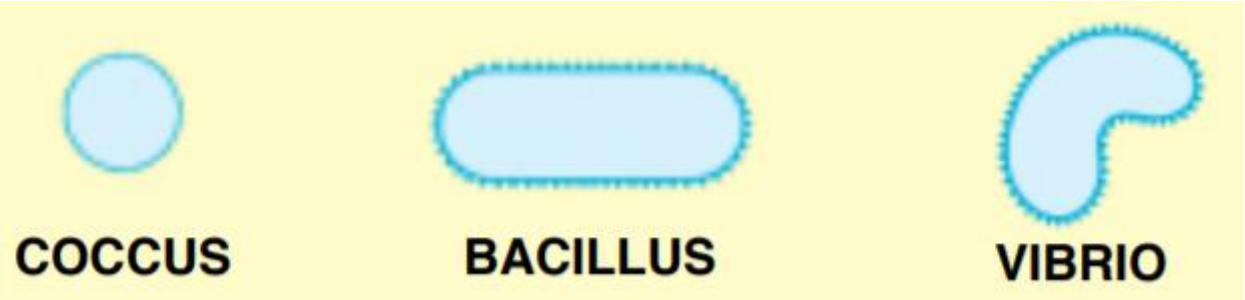


Figure 4.8: The three most common shapes are the rod (bacillus), the sphere (coccus), and the spiral type (vibrio).

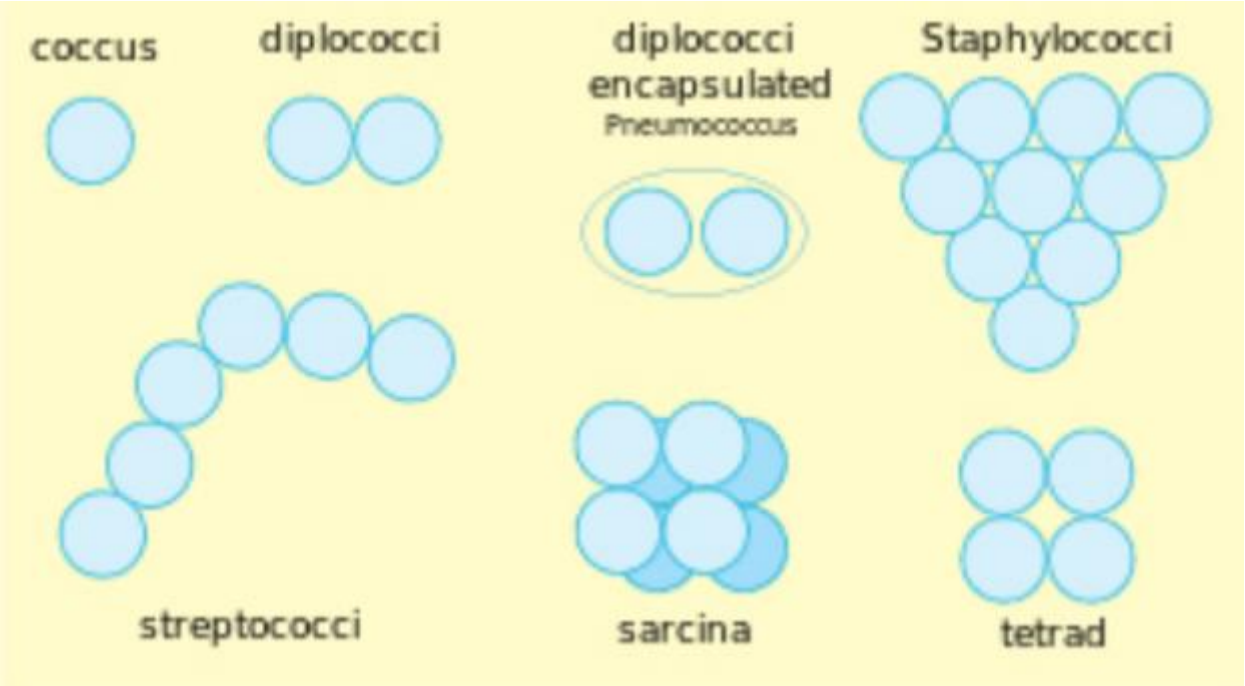


Figure 4.9: Arrangement is the manner by which groups of bacteria appear together. Some common arrangement types are paired (diplo), grape-like clusters (staphylo) or chains (strepto).

Work in groups on these problems. You should try to answer the questions without referring to your textbook. If you get stuck, try asking another group for help.

- Did you inoculate your broth, slant, and plate properly? How do you know?

- What is aseptic technique and why do we use it?

- You inoculate a plate from a plate, you see no growth the next day. You know the plate had live bacteria on it. What went wrong?

- What is the difference between a slant and a slant/deep? NOTE: do not just define the two terms, write an actual sentence describing the difference.

- Which plate, the one inoculated from a broth or the one from a plate, would you expect to see more bacteria on? Why?

Jane was very careful in performing her Petri dish inoculation. When she examined her plate during the next lab period, a fuzzy white colony was growing on her plate, but not along her inoculation streak. What happened? Where did this colony come from?

_____

_____

_____

_____

Jeffrey, Jane's partner, inoculated a tryptone broth tube with *E. coli.* When he examined his broth tube during the next lab period, there was no growth at all. What could have gone wrong with his experiment?

_____

_____

_____

Five bonus point will be granted for the best answer after discussion!!!! Good Luck !!!!

Chapter 5: Microbial phototrophs: Algae & Cyanobacteria

Objectives

- List the major group of microbial phototrophs
- Recall the environmental and clinical significance of these organisms
- Describe the characteristics, structure and by-product of microbial phototrophs

Introduction

Prokaryotic Organisms - Cyanobacteria have been around for about 3.5 billion years and have survived conditions ranging from volcanoes to the Ice Age. Cyanobacteria, formerly known as blue-green algae, are photosynthetic microscopic organisms that are technically bacteria. They were originally called blue-green algae because dense growths often turn the water green, blue-green or brownish-green. These algae are found in all lakes and are a natural part of the lake ecosystem. Unfortunately, high nutrient concentrations can promote a population explosion of these organisms and result in algal blooms, especially during warm weather.

Cyanobacteria belong to Domain Bacteria. These microscopic organisms lack nuclei. They were once classified as algae because they have chlorophyll a. This explains why they are often referred to as the blue-green algae. Cyanobacteria do not contain chloroplasts. Over a 1,000 species of cyanobacteria have been reported. They are present in almost all moist environments. We will look at two examples of cyanobacteria: *Anabaena* (**Figure 5.1**) *Oscilltoria* (**Figure 5.2**). Anabaena is a filamentous cyanobacteria that exists as plankton.

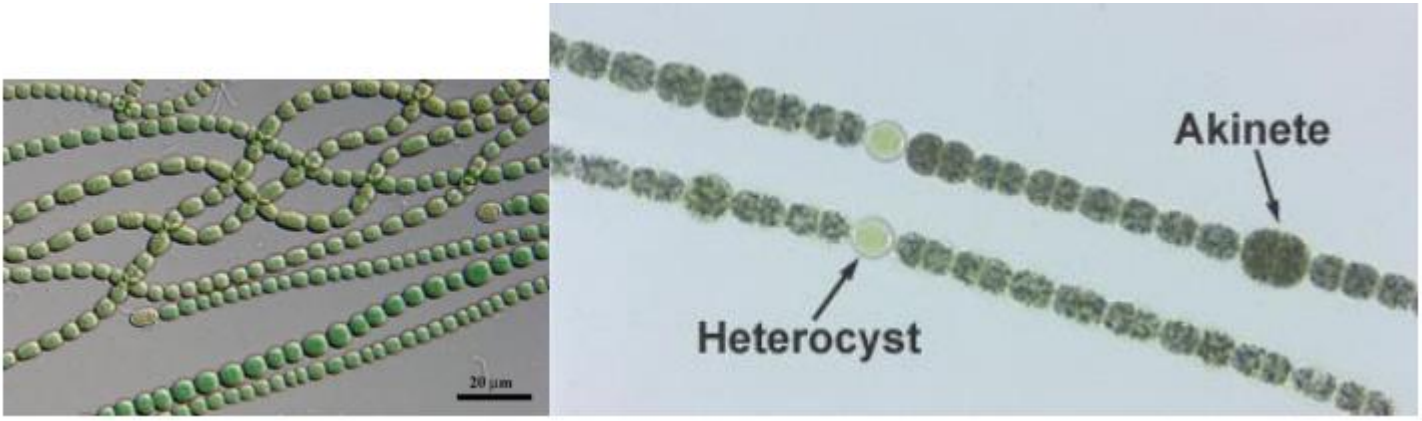


Figure 5.1: Structure of *Anabaena*

It is known for its nitrogen fixing abilities. Anabaena can develop akinetes (thick-walled resting cells that can survive in sediments for many years). It possesses heterocysts, which, during times of low environmental nitrogen, supply neighboring cells with fixed nitrogen in return for the products of

photosynthesis. About one cell out of every ten will differentiate into a heterocyst that can no longer perform photosynthesis. This separation of functions is essential because the nitrogen fixing enzyme in heterocysts, nitrogenase, is unstable in the presence of oxygen. Some Anabaena form symbiotic relationships with certain plants, such as the mosquito fern. Anabaena are one of four genera of cyanobacteria that produce neurotoxins, which are harmful to local wildlife, as well as farm animals and pets. Production of these neurotoxins is assumed to be a part of its symbiotic relationships, protecting the plant from herbivores.

Oscillatoria is a genus of filamentous cyanobacterium which is named for the oscillation in its movement. Filaments in the colonies can slide back and forth against each other until the whole mass is reoriented to its light source. It is commonly found in watering-troughs waters, and is mainly blue-green or brown-green. Oscillatoria is an organism that reproduces by fragmentation.

Figure 5.2: Structure of *Oscillatoria*

Nostoc: An organism older than the dinosaurs could be living in your lawn! Nostoc is not easy to control. Within the category of "What is it?" lives a strange-looking organism called by many names. Referred to as star jelly or star slime, these names harken back to beliefs that this organism had fallen from the dust of shooting stars, whereas the name witch's butter speaks to its slippery nature. Following a period of rain, Nostoc may appear suddenly in lawns, pastures, paved surfaces, stones or roofs, which may be why people associated it with having celestial origins, seemingly coming out of nowhere.

Figure 5.3: The jelly-like blobs of green are colonies of Nostoc, a phylum of bacteria that get their energy through photosynthesis. This specimen was found growing in a lawn. The tan granules are grains of sand. Photo by MSU Extension.

Are all Blooms Toxic?

Not all blue-green algae or algal blooms are toxic. The reasons algae produce toxins at any given time are not well understood. Standard monitoring techniques cannot predict when a bloom has toxins in it. While

heavy growths of blue-green algae often show detectable levels of toxins, only the most intense blooms like those above create a potential for significant toxin exposure for humans and animals. By far the most common and best known toxins are the various forms of microcystins.

How Toxic are these Compounds?

Toxic reactions depend on a number of factors, including the degree of exposure (how much water is swallowed, how long the water is in contact, etc.), the concentration of toxins in the water, and, the sensitivity of the individual. Toxins are not readily absorbed through the skin and it is not clear if health problems can arise from inhaling water droplets with toxins. Though small amounts can cause mild reactions in sensitive individuals, significant human illness has been only rarely reported. However, severe reactions and death of pets or livestock drinking contaminated water have been reported from many locations outside of Maine.

Are these Toxins found in Maine Lakes?

Since 2008, DEP has been measuring concentrations of microcystins in lakes that regularly support algal blooms and in lakes considered to free of blooms. By sampling both types of lakes, biologists are confident that we are establishing a dataset that will characterize all Maine lakes and provide insight on how toxin concentrations compare to EPA's guidelines. In addition, these data will form the basis for how the State of Maine's Department of Health and Human Services, Center for Disease Control will create an advisory specific to our lakes.

Preliminary results indicate that most Maine lakes that do not produce algal blooms are safe to drink (after disinfection) and recreate in. In contrast, lakes that regularly produce algal blooms are most likely to produce toxins, the highest concentrations of which are found in the scums that accumulate along the shoreline; these scums may be 100-1000 times the levels of concern issued by EPA. Swimming areas and deep waters often produce toxin concentrations that exceed EPA guidelines, but in the range of 1-10 times EPA's guidelines. Results also indicate that toxins are produced later in the bloom period, when cell numbers are most dense and cells are beginning to die.

How to Avoid Problems?

While most adults will avoid green discolored water, a hot day can lure children and pets into the water. The following list of recommendations will help you minimize risk.

- Do not accidentally ingest or drink lake water during a bloom. Well maintained domestic water treatment systems may make lake water safe to drink by removing bacteria and parasites.
- If you shower with lake water, keep showers brief because breathing toxins in shower mist could cause health issues. Do not swim, water ski, or boat in areas where algae are visible (e.g., pea soup, floating mats, scum layers, etc.), where water is discolored, or where musty odors are present.
- Rules of thumb: if you are standing in water chest deep (4-5 feet) and you can't see your toes because the water is so green, you should get out; if you are looking into water that is 4-5 feet deep and can't see the bottom of the lake because the water is so green, you should not to go in.

- Because algal scums along the shoreline have the highest concentrations of toxins, do not let children play in water that is discolored, where you see mats of algal material, foam, or where musty odors are present. Do not allow pets or livestock swim or drink water from these areas. Rinse off with fresh water and soap if available, as soon as practical if exposed to water that has dense algae present. This will reduce skin exposure for humans and pets.

Examine and sketch the given specimen under 10X and 40X magnification. Also take a snap using your mobile for both conditions.

Specimen_____

Magnification_____

Specimen_____

Magnification_____

Specimen_____

Magnification_____

Specimen_____

Magnification_____

Examine and sketch the given specimen under 10X and 40X magnification. Also take a snap using your mobile for both conditions.

Specimen_____ Specimen_____

Magnification_____ Magnification_____

Specimen_____ Specimen_____

Magnification_____ Magnification_____

Algae: Alga is a term that describes a large and incredibly diverse group of phototrophs, photosynthetic lifeforms. Multicellular examples of algae include the giant kelp and brown algae. Unicellular examples include diatoms, Euglenophyta and Dinoflagellates.Most algae require a moist or watery environment; hence, they are ubiquitous near or inside water bodies. Anatomically, they are similar to another major group of photosynthetic organisms – the land plants. However, that is where the differences end as algae lack many structural components typically present in plants, such as true stems, shoots, and leaves.

Furthermore, they also do not have vascular tissues to circulate essential nutrients and water throughout their body. Recent developments in science and technology have enabled algae to be used as a source of fuel. Global demand for petroleum products and declining environmental health have prompted the use of eco-friendly alternatives such as algal biofuel. Hence, algae fuel is an increasingly viable alternative to traditional fossil fuels. It is used to produce everything from "green" diesel to "green" jet fuel. It is similar to the other biofuels made from corn and sugar cane.

Characteristics of Algae

Specific general characteristics of algae are common to plants as well as animals. Algal cells are eukaryotic. For instance, algae can photosynthesize like plants, and they possess specialized structures and cell-organelles, like centrioles and flagella, found only in animals. The algal cell walls consist of mannans, cellulose and Galatians. Listed below are some of the general characteristics of algae.

- Algae are photosynthetic organisms
- Algae can be either unicellular or multicellular organisms
- Algae lack a well-defined body, so, structures like roots, stems or leaves are absent
- Algaes are found where there is adequate moisture.
- Reproduction in algae occurs in both asexual and sexual forms. Asexual reproduction occurs by spore formation.
- Algae are free-living, although some can form a symbiotic relationship with other organisms.

Volvox is a type of green algae. It forms spherical colonies of up to 50,000 cells. They live in a variety of freshwater habitats. Each mature Volvox colony is composed of numerous flagellate cells embedded in the surface of a hollow sphere. The cells swim in a coordinated fashion, with distinct anterior and posterior poles. The cells have eyespots which enable the colony to swim towards light. The spheres will break up with advanced age. The generative cells then grow into new colonies.

Ulothrix is a filamentous green alga, generally found in fresh and marine water. Its cells are normally as broad as they are long, and they thrive in the low temperatures of spring and winter. They become attached to surfaces by a modified holdfast cell.

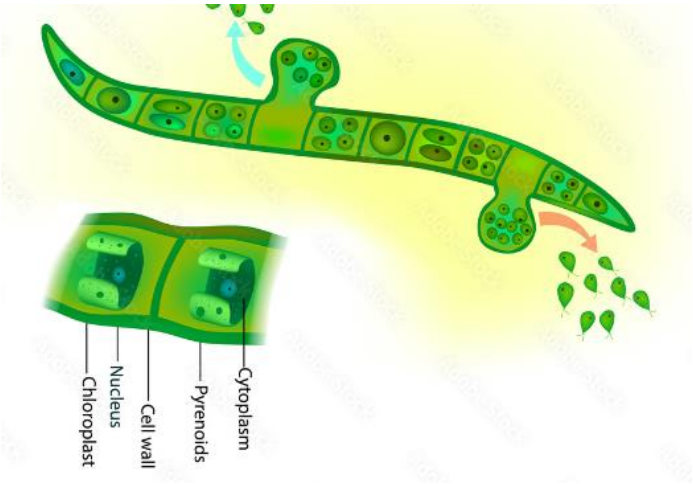


Figure 5.4: *Ulothrix*

Spirogyra is a filamentous green alga of the, named for the helical or spiral arrangement of the chloroplasts. It is commonly found in freshwater areas. Spirogyra measures approximately 10 to 100µm in width and may stretch centimeters long. This particular algal species, commonly found in polluted water,

is often referred to as "pond scum". The cell wall has two layers: the outer wall is composed of pectin that dissolves in water to make the filament slimy to touch while the inner wall is of cellulose. The cytoplasm forms a thin lining between the cell wall and the large vacuole it surrounds. The chloroplasts are ribbon shaped, serrated or scalloped, and spirally arranged. In spring *Spirogyra* grows under water, but when there is enough sunlight and warmth they produce large amounts of oxygen, adhering as bubbles between the tangled filaments. The filamentous masses come to the surface and become visible as slimy green mats.

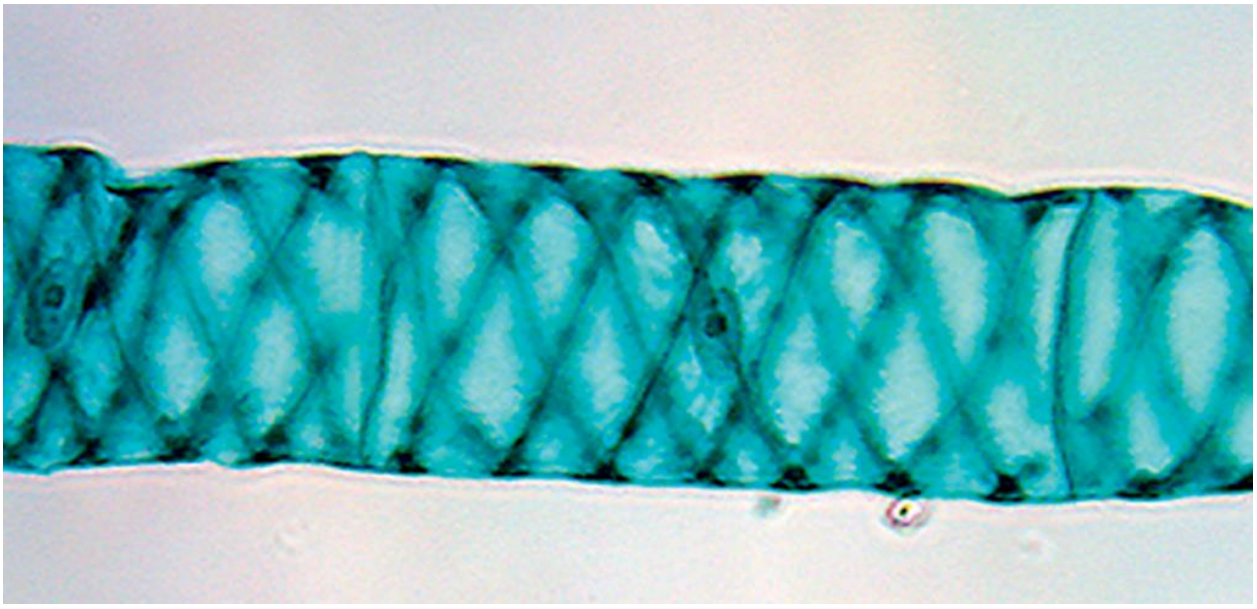
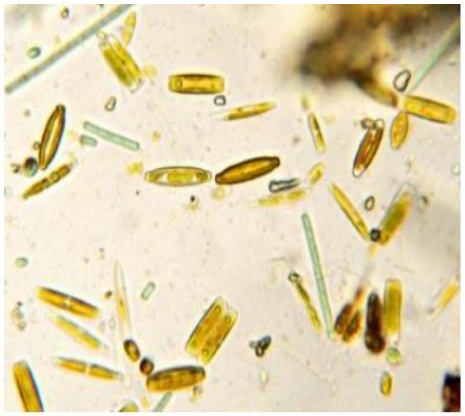
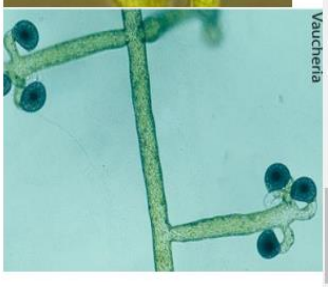


Figure 5.5: *Spirogyra*

Diatoms are a major group of golden brown algae, and are one of the most common types of phytoplankton (microscopic plants). Diatoms are primary producers within the food chain. Most diatoms are unicellular, although they can exist as colonies in the shape of filaments or ribbons, fans, zigzags, or stellate colonies. Diatom cells are characteristically encased within a unique cell wall made of silica (hydrated silicon dioxide) called a frustule. These frustules show a wide diversity in form, but usually consist of two symmetrical sides with a split between them, hence the group name.



Vaucheria is another **golden brown algae**. *Vaucheria* exhibits apical growth from the tip of filaments forming mats in either terrestrial or freshwater environments. Its filaments form coenocytes with a **large central vacuole** pushing against the surrounding cytoplasm; the vacuole extends along the entire filament except for the growing tip. The chloroplasts are located on the periphery of the cytoplasm with the nuclei aggregating toward the center near the vacuole.



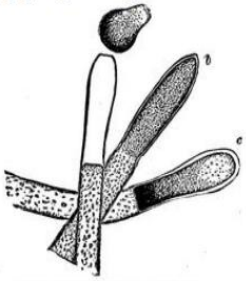


FIG. 1.—GROWTH OF GONIDIA.

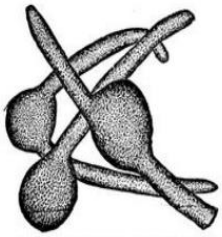


FIG. 2.—GONIDIA GERMINATING.

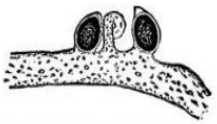


FIG. 3.—ANTHERIDIUM AND OOSPORES. V. SESSILIS.

FIG. 4.—ANTHERIDIUM AND OOSPORES. V. RACEMOSA.

Figure 5.6: Different types of Diatoms

1. How are algae different from traditional plants?

2. State the general characteristics of algae

3. Are algae toxic to human?

4. Why are algae important and how they are useful?

5. What are algal blooms?

Examine and sketch the given diatoms under 10X and 40X magnification. Also take a snap using your mobile for both conditions.

Specimen_____

Magnification_____

Specimen_____

Magnification_____

Chapter 6: The Protists - Eukaryotic Organisms Kingdom Protista

Prokaryotic and Eukaryotic Cells As per our knowledge, the building blocks of life are cells. Prokaryotic cells are those cells that do NOT have a nucleus. They mostly include bacteria and archaea. These cells do not have membrane-bound organelles. Eukaryotic cells are those that have a true nucleus. That would include plant, animal, algae, and fungal cells. As you can see, to the left, eukaryotic cells are typically larger than prokaryotic cells.

In this lab**,** we will look at examples of both prokaryotic and eukaryotic unicellular organisms, most of which are commonly found in pond water. When examining pond water under a microscope, the unpigmented, moving microbes will usually be protozoans. Greenish or golden-brown organisms will typically be algae. As we discussed in previous chapter, microorganisms that are blue-green will be cyanobacteria. Cyanobacteria are photosynthetic bacteria (once thought to be algae, so they are sometimes referred to as blue-green alga) that are generally blue-green in color and, in some species are capable of nitrogen fixation. In this chapter, we will focus on protozoans. Protozoans are a large group of single-celled, usually microscopic, eukaryotic organisms, such as amoebas, ciliates, flagellates, and sporozoans. Algae are unicellular or multicellular organisms (formerly classified as plants) that have chlorophyll but lack true stems, roots, and leaves. Algae include the seaweeds, diatoms, and spirogyra (as we learned in previous chapter).

The Protists - Eukaryotic Organisms Kingdom Protista: Single-celled, eukaryotic organisms are called protists. They are grouped together in Kingdom Protista. Those protists that are animal-like are put in Subkingdom Protozoa, and the protists that are plantlike belong to Subkingdom Algae. The Protists – Subkingdom Protozoa Members of Subkingdom Protozoa, known as the **protozoans**, have a cell membrane (the pellicle) but lack cell walls. The nuclei are distinct, and the cells often have specialized organelles such as contractile vacuoles, mitochondria, flagella, and cilia. All protozoans produce cysts which are resistant dormant stages that enable the protozoans to withstand drought, heat, and freezing. The protozoans we will look at today include the following: ***Amoebas, Paramecium*** *and* ***Plasmodium.***

Amoebas are unicellular heterotrophs (they must eat food, as all other animals). They obtain food through phagocytosis, utilizing pseudopods to entrap their meals. Amoebas' most recognizable features include one or more nuclei and a simple contractile vacuole to maintain osmotic equilibrium. Like other eukaryotic organims, they reproduce through asexually via mitosis and cytokine.

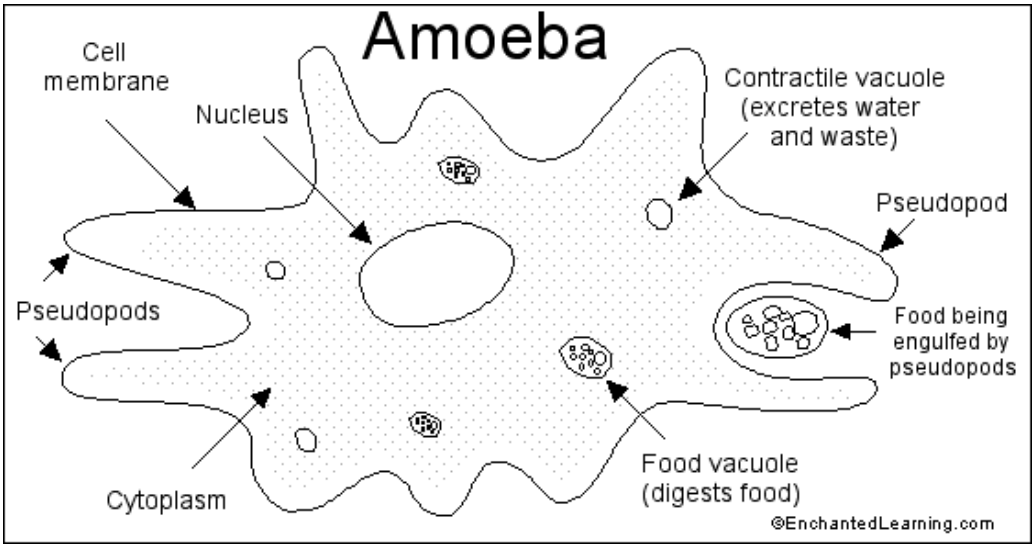


Figure 6.1: Structure of *Amoeba*

Parmecium are unicellular, ciliated protozoans (microscopic animals). Simple cilia cover the body, which allow the cell to move with a synchronous motion (like a caterpillar) at speeds of approximately 12 body lengths per second. There is also a deep oral groove containing inconspicuous tongue-like compound oral cilia used to draw food inside. They generally feed on bacteria and other small cells. Osmoregulation is carried out by a pair of contractile vacuoles, which actively expel water from the cell absorbed by osmosis from their surroundings. Paramecia are widespread in freshwater environments.

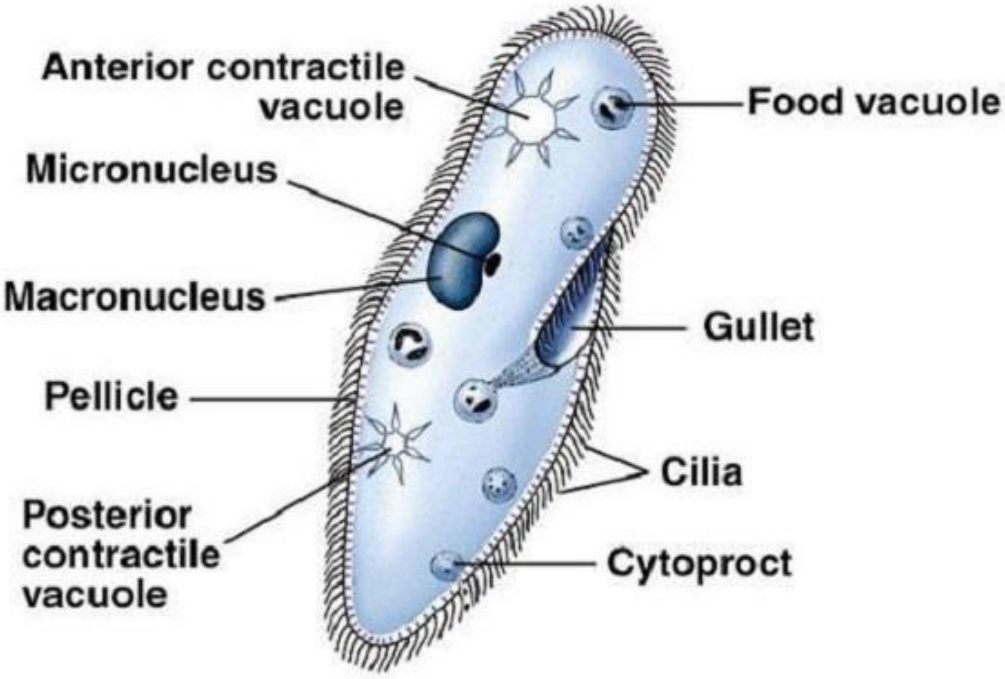


Figure 6.2: Structure of *Paramecium*

Euglena is a genus of unicellular, freshwater organisms that are very common in ponds and small bodies of water, especially if they are rich in nutrients and consequently high in algae (aka 'pond scum'). As noted

below, *Euglena* itself is sometimes photosynthetic and is a component of the green sludge in such ponds. But at other times it is non-photosynthetic and is a component of the diverse group of organisms that are eating the green sludge or perhaps eating the other things that eat the green sludge.

Euglena are unicellular protozoans that almost always have chloroplasts. Although they photosynthesize (autotrophy), Euglena can also eat food by heterotrophy (like animals). They use a flagellum for locomotion.

Euglena is a unicellular organism with a complex internal structure that includes a contractile vacuole that can expel water and a red 'eyespot'. Photosynthetic forms contain a chloroplast. They possess two flagellae, one long, one short, that can allow the organisms to move. *Euglena* are also able to move by means of changing its shape (see video links). Outside the cell membrane is a flexible, protein-based structure called a pellicle. Although not generally considered a cell wall, it has similar functions in providing some rigidity and strength that the membrane cannot provide. However the pellicle is much more flexible than most cell walls and allows for the change in form that is often seen in *Euglena* motion.

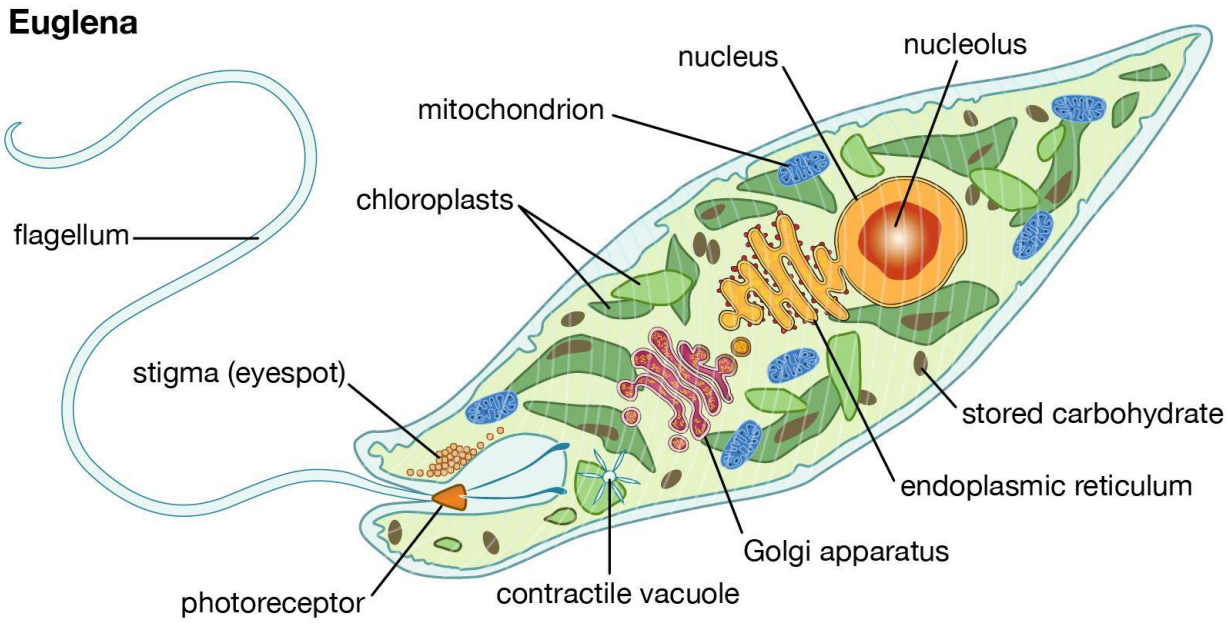


Figure 6.3: Structure of *Euglena*

What are two major visual features of Paramecium?

Draw and describe the internal structure of *Euglena* below: Is *Euglena* Plant or Animal? Why?

Examine and sketch the given specimen under 10X and 40X magnification. Also take a snap using your mobile for both conditions.

Specimen_____

Magnification_____

Specimen_____

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Chapter 7: The Fungi

Fungi belong to the kingdom Fungi. They include large mushrooms and molds as well as microscopic yeasts. Unlike plants, fungi do not perform photosynthesis instead, they obtain nutrients through absorption from organic matter in their environment. Fungi play important roles in ecosystems as decomposers, breaking down dead organic material and recycling nutrients. They also form symbiotic relationships with plants, helping in nutrient uptake and enhancing plant growth. Overall, fungi have a major influence on human society and are essential to the functioning of ecosystems. The diagram of fungi clearly shows fungal structure which makes studying the functions of fungi easier.

A diagram of fungi can be a helpful visual aid to understanding the various roles played by fungi in ecosystems and human life. The simple diagram of fungi with labeling shows the basic structure of a fungus. The fungus is made up of a mass of thread-like structures called hyphae. The hyphae are branched and interconnected, forming a network called the mycelium. **Figure 7.1 shows the t**he labeled diagram of fungi.

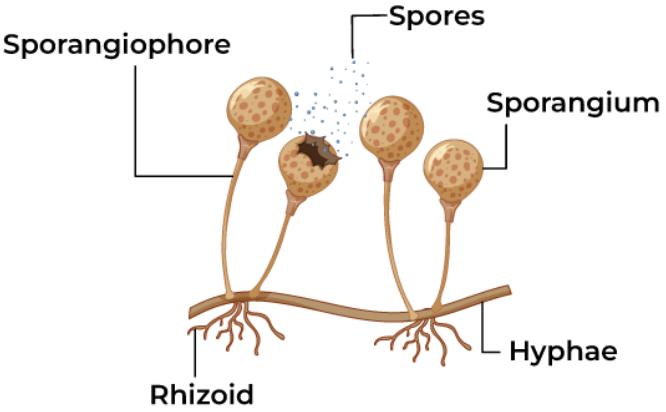

Figure 7.1: A typical diagram of Fungi

As shown in **Figure 7.2**, In this lab you observed a variety of Fungi. You will be expected to classify each organism into the appropriate phylum and understand the role it plays in its environment. As you review the images on this page, you should be able to identify the structures listed in bold face in your manual, know their functions and recognize reproductive structures as involved in sexual or asexual reproduction.

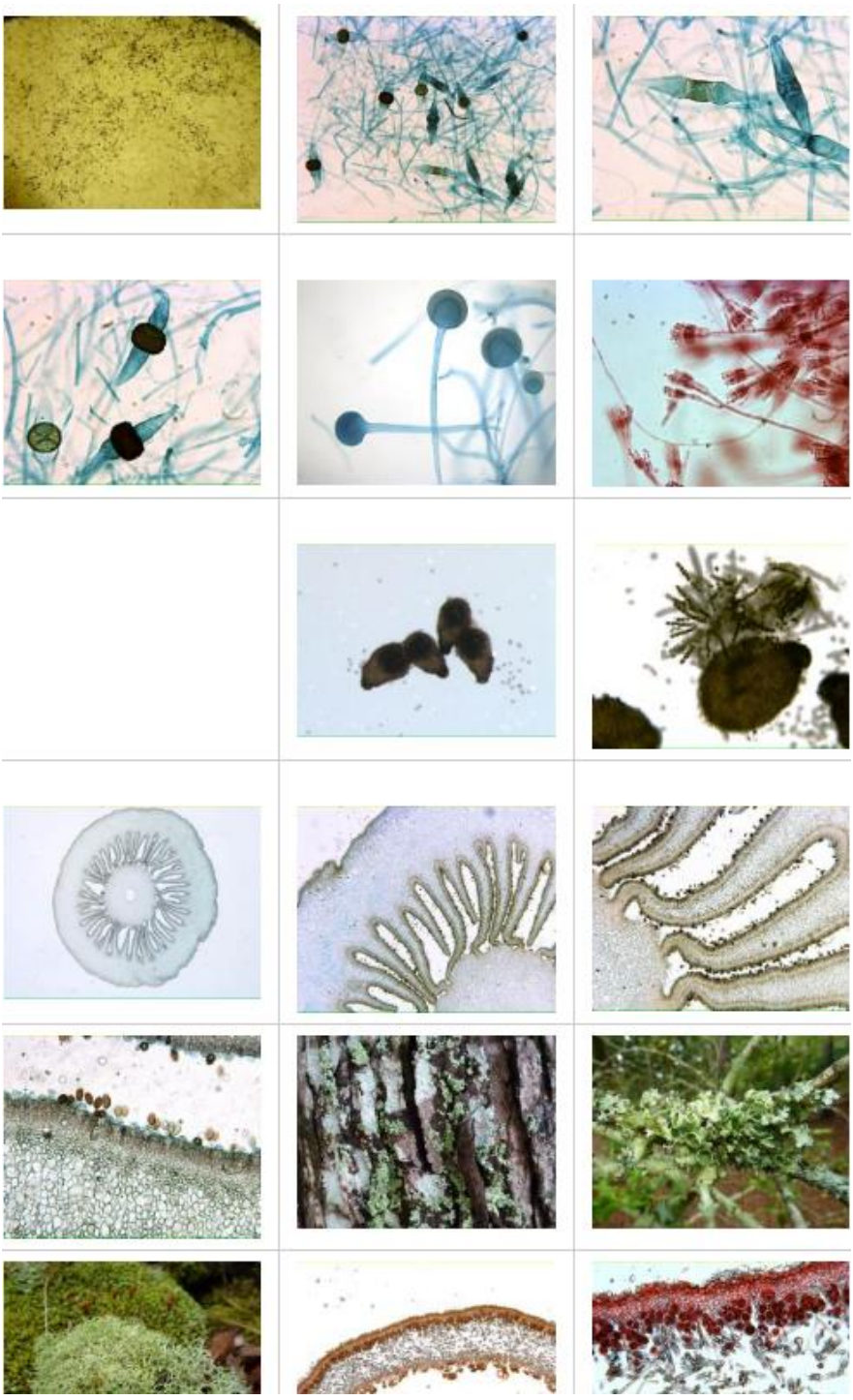

Figure 7.2 Different types of Fungi

The structure of fungi diagram shows the follow features:

- Cellular Composition: Fungi are eukaryotic organisms with nucleus enclosed within nuclear membrane.
- Cell Walls: Unlike plants, fungi have cell walls made of chitin, a tough, structural polysaccharide.
- Hyphae: The body of a fungus is typically composed of thread-like filaments called hyphae. These hyphae may be septate (divided by cross-walls called septa) or non-septate (coenocytic).

- Mycelium: A network of hyphae forms the mycelium, which is the main body of the fungus. Mycelium grows through the substrate, absorbing nutrients from its surroundings.
- Reproductive Structures: Fungi reproduce through the production of spores. These spores can be produced sexually or asexually and are dispersed to new locations for reproduction.
- Fruiting Bodies: In some fungi, specialized structures called fruiting bodies are formed for spore production and dispersal. Examples include mushrooms, puffballs, and yeast colonies.
- Rhizoids and Root-Like Structures: Some fungi have rhizoids or other root-like structures that help in attachment and nutrient absorption.

Functions of fungi include:

- Break down dead organic matter, recycling nutrients.
- Form beneficial relationships with plants (mycorrhizae) enhancing nutrient uptake.
- Used in food production for fermentation of bread, cheese, beer.
- Source of antibiotics, antifungals, and other pharmaceuticals.
- Enzyme production, biofuel production, and other industrial applications.
- Contribute to soil structure and fertility through decomposition.
- Maintain balance in ecosystems by regulating populations and nutrient cycling.

Conclusion – Diagram of Fungi

In conclusion, the diagram of fungi illustrates the structure of fungi which highlights the various roles of fungi in both natural ecosystems and human society. Fungi show diversity and importance by playing an important part in nutrient cycling and decomposition. Fungi also contribute to food production, medicine, and biotechnology. Studying the roles and structure of fungi is essential for recognizing their impact on our environment and lifestyles.

Make some important question and try to explain the hypothesis behind the question you ask yourself. (5 bonus points)

Examine and sketch the given specimen under 10X and 40X magnification. Also take a snap using your mobile for both conditions.

Specimen_____

Magnification_____

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Chapter 8: Bacterial Staining (Simple, negative and Gram Stain)

Objectives

- To understand and prepare a simple stained slide
- To explain the basis for and advantage of simple staining
- To understand and prepare a negatively stained slide
- To explain the basis for and advantage of negative staining
- To understand and prepare a gram-stained slide
- To explain the basis for and advantage of gram staining

Introduction

Almost all bacteria very small and very clear which cause it is very difficult to view under a microscope without staining. Therefore, you must firmly attach your bacteria to a glass slide before staining them. There are mainly two main things to be considered whenever you prepare for staining slide for any type of bacteria.

1. The bacteria must be evenly and lightly dispersed. If there are too many bacteria on the slide they will form a big glob and you will not be able to see the morphology of the individual cells. Large blobs of cells also do not stain properly and could yield erroneous results from the improper staining.

2. The bacteria need to be firmly attached to the slide so they are not washed off during the staining procedures. All procedures that attach the bacteria to the slide result in some morphological changes. The cells typically shrink in size and will exhibit some changes in shape and extra-cellular matrices.

You will be preparing slides for staining from both broth and agar surfaces. While the goals are the same for both, evenly and lightly dispersed cells firmly adhered to the slide surface, the techniques are slightly different. Staining is as much art as science. It will undoubtedly take you several tries before you are successful.

Materials: Clean glass slides, Inoculating loops or needles, Sterile water, Marking pen,Assorted broth and plate cultures

Safety Considerations

Be careful of aerosols when transferring bacteria from your loop to the slide. The loop is very flexible and it is easy to zing off a loop-full of organisms. Do not assume your organism is dead. Heat or methanol fixation is not guaranteed to kill the organism. Dispose of your completed slides in the disinfectant bucket at your bench.

General Considerations

You are striving for a light suspension of cells that will leave a faint cloudy deposit on your slide. You have lots of room on your slide; use it! It helps to initially draw a circle on the bottom of the slide so you know where to look for your smear. It is very easy to get confused which side of the slide your smear is on. Be sure to label the far edge of the slide. Do this consistently on the same end of the slide to help orient your slide.

Be patient and take the time to let your slide air dry before proceeding with adhering it to the slide. If your slide is wet and you heat fix it, the bacteria will boil and the cellular morphology will be lost. If your slide is wet and fix it in methanol, it will most likely wash off the slide. Smears that are too thick will most likely wash off the slide regardless of the fixation method.

Simple Staining

Smear from Broth

Broth cultures are usually easier to work with because the cells are already diluted in the broth. Be sure to carefully mix the culture tube to suspend the bacteria in the broth.

1. Label your slide. Aseptically transfer a loop-full of organism onto the center of your slide.
2. Use the flat part of the loop to smear the broth drop around the slide. Use a spiraling, circular motion to spread out the drop. Because the broth is full of protein, the smear will usually stay spread out and not bead up on the surface of the slide.
3. Set the slide aside to air dry. This will take several minutes at least. Do not rush this step.

Smear from Plate

You can scoop a lot of organisms off with your loop. You may want to use an inoculating needle to transfer your organism to the slide. Be sure to use sterile water to dilute your samples. Regular tap water or the de-ionized water in your rinse bottles are often contaminated with bacteria.

1. Label your slide. Aseptically transfer a loop-full of sterile water to the center of the slide.
    - This serves to both dilute your bacteria and give you something to spread around.
2. Pick a well-isolated colony.
3. Prick it with your sterile needle, or slightly scoop the edge of the colony with your sterile loop.
4. Place your needle/loop in the center of the drop and with a spiraling circular motion spread the bacteria on the slide.

5. Set the slide aside to air dry. This will take several minutes at least. Do not rush this step.

Fixation: The fixation procedure is the same regardless of smear source, plate or broth. There are two methods of adhering your bacteria to the slide, heat fixation or methanol fixation. Heat fixing is only used with BSL1 organisms. The organisms we will be working with are BSL2, so you will need to use the methanol fixation technique. Heat fixing the slide can create aerosols and with BSL2 organisms, we need to prevent this as much as possible. Methanol fixation causes fewer changes in cellular morphology and creates no aerosols.

Please be careful when working with the methanol, if you forget you have fixed it with methanol and your slide isn't totally dry, the remaining methanol will catch on fire.

Methanol Fixing: Be sure your slide is totally dry. Set it on the staining rack over the sink.

1. Carefully flood the slide with 95% methanol. Let it sit for two minutes.
2. Tilt the slide and pour off the methanol.
    - Touch the edge of the slide to a paper towel to wick off the excess methanol.
3. Set the slide aside to air dry before staining.

1. Working in pairs, label each slide and draw a circle on the center of the slide with a wax pencil which is provided at your table, DO NOT use a sharpie, this will keep the bacteria concentrated in one area on the slide.

2. Prepare an emulsion on each slide:

- If you are taking a bacteria from a plate, place a small drop of water on a slide and aseptically add bacteria.
- If you are taking a bacteria from a broth, place 3-6 loopfuls of bacteria onto a slide with no water added.

3. You and your lab partner will need to prepare the following slides:

- 3 slides of *Staphylococcus epidermidis* (one for simple stain, one for gram stain, one as back-up)
- 3 slides of *Pseudomonas aeruginosa* (one for simple stain, one for gram stain, one as back-up)
- 2 slides of S. epidermidis and P. aeruginosa mixed together (one for gram stain, one as back-up)

4. Mix gently until you get an even cloudy mixture (should look like skim milk

**If you mix too aggressively, you will lose the bacterial morphology.*
5. Allow the slide(s) to air dry on the slide warmer. While the slides are drying, start your negative stain.

6. Once the liquid has completely evaporated, heat fix by passing the slide through a flame three times.

Simple Stain: Simple stains are just that - add one stain to a fixed smear slide, let it sit, rinse it off, let it dry, and view. It is a quick procedure for determining the presence and morphology of bacteria in clinical samples such as stool and discharges.

Methylene blue is used to determine the morphology of fusiform and spirochetes in oral infections. It is also the stain of choice for identifying the metachromatic granules in *Corynebacterium diphtheriae*. The granules will stain a distinctly deeper blue than the surrounding blue bacteria. Other species of *Corynebacterium* do not have the metachromatic granules. Any basic dyes, such as methylene blue, crystal violet, malachite green, or safranin work well.

Basic (cationic or positively charged) dyes bind to negatively charged components in the cell membrane and cytoplasm.

Materials: Methylene blue, Safranin, Crystal violet, Malachite Green, Staining racks, Prepared smear slides

General Considerations: Staining is part art and part science. There are no hard and fast rules for staining and rinsing times. The times listed are suggestions that usually work well. You will need to experiment with what works for the bacteria you have and the techniques you use.

Simple Stain Procedure

1. Place your carefully prepared fixed smear slides on the stain rack over the sink.
    - Do one slide at a time.
    - Cover the smear with any of the basic dyes available to you.
    - You only need enough dye to cover the smear. The stain should not drip off the slide.
2. Let the stain sit for 1-5 minutes.
3. Using the clothespin, grab the long end of the slide, tilt the slide over the sink and rinse the stain off with a stream of water from the wash bottle.
    - Be sure to spray above the smear and let it dribble down.
    - If you spray directly on the smear you are liable to wash the smear off the slide.
    - Rinse till the water runs clear or is only slightly colored.
4. Touch the edge of the slide to a paper towel to remove excess water. You can now let it air dry. Alternately you can dry it with blotting paper by placing it in the blotting paper book and pressing lightly. While this method is quicker, you can also blot off a poorly adhered smear.
5. View your slide under oil immersion and record your observations in your lab book.
6. Discard your used stained slides in the disinfectant bucket in the sink.

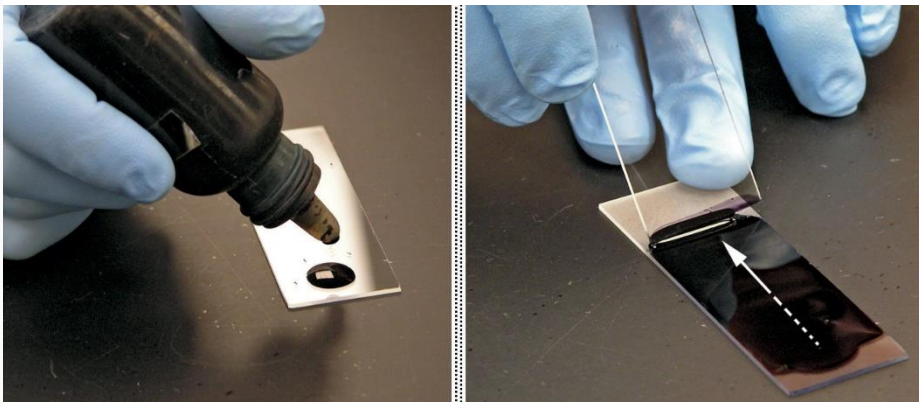
Negative Staining

Organisms

- *Bacillus* (slants or plates)
- *Klebsiella* (milk broth)

Materials

- 2 clean slides
- Loop
- Staining tray
- Nigrosin
- Coverslip
- Bunsen burner and striker



A B

Figure 8.1: Preparing the negative stain smear. Apply a drop of nigrosine near the end of slide and mix bacteria on it. Use the second clean slide to spread the nigrosine.

Figure 8.2: *Bacilus* and *Klebsiella* were incubated on a drop of nigrosin to prepare this slide. The cells appear as bright as bright white shapes against a dark background

Negative Staining Procedure

Label your slide. If you are working from a broth culture, place a loop-full of organisms about three fourths of the way on the left side of the slide. If you are working from a plate culture, add a drop of sterile water to the slide and dilute your organism in the drop without spreading the drop.

1. Place your slide on the staining tray or benchtop.
2. Add one drop of nigrosin to one end of the slide (not in the middle) **Figure 8.1 A**
3. Aseptically add one loopful of *Klebsiella* to the drop of nigrosin.
4. Flame your loop.
5. Aseptically add a small amount of *Bacillus* to the same drop of nigrosin in order to create a mixture of the two organisms.
6. Pick up the second clean slide. You will use this slide to spread the smear. Hold the clean slide at about a 30-degree angle to the slide.
7. Start on the end of the slide away from the dye. Slide the clean slide toward the nigrosin drop until the dye runs along, the second slide edge and across the width of the slide.
8. In one single motion, pull the second slide quickly down the length of the slide **Figure 8.1 B** This should result in a long, dark smear with varying thicknesses along the entire length of the slide.
9. Allow the slide to air-dry.

10. When the slide has air-dried, cover the smear with a coverslip and view under oil immersion.

- Record your observations in your lab book.
- Discard your used stained slide in the disinfectant bucket.

Gram Staining: This technique was proposed by Christian Gram to distinguish the two types of bacteria based on the difference in their cell wall structures. The gram-positive bacteria retain the crystal violet dye, which is because of their thick layer of peptidoglycan in the cell wall.

This process distinguishes bacteria by identifying peptidoglycan that is found in the cell wall of the gram-positive bacteria. A very small layer of peptidoglycan is dissolved in gram-negative bacteria when alcohol is added.

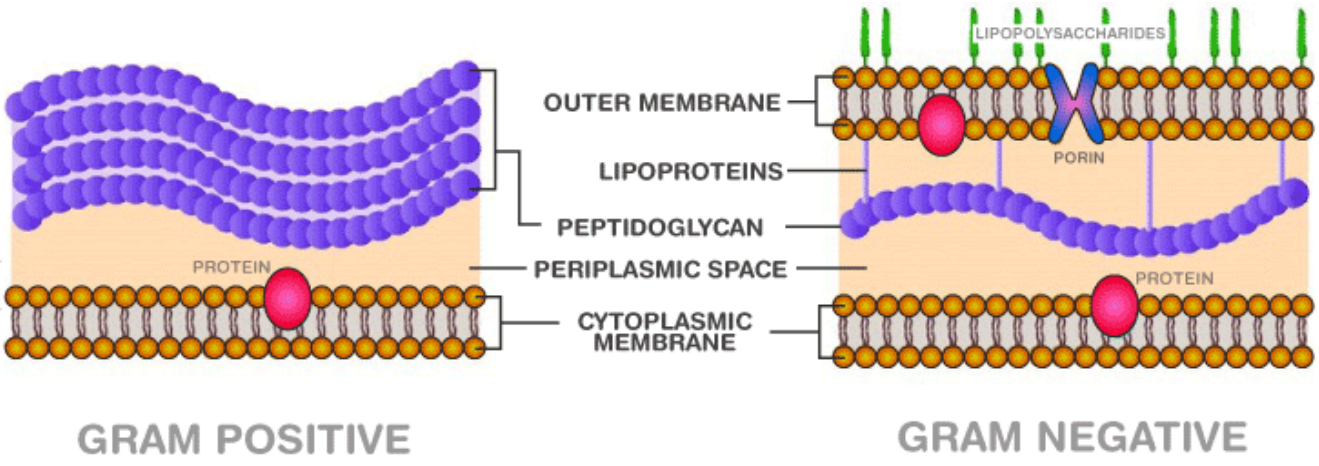


Figure 8.3: Difference between Gram-positive and gram-negative bacteria. Gram-negative bacteria have cell walls with thin layers of peptidoglycan (10% of the cell wall) and high lipid (fatty acid) content. This causes them to appear red to pink under a Gram stain.

Gram stain is a common laboratory test that can help diagnose the presence of a bacterial infection quickly.

Healthcare providers often order it alongside a bacteria culture to diagnose the type of bacterium causing the infection. A Gram stain is a laboratory test that checks for bacteria at the site of a suspected infection or in certain bodily fluids. A medical laboratory scientist processes the Gram stain, which gives relatively quick results, so healthcare providers can know if bacteria are present, and, if so, the general type(s). This can help guide further identification tests and treatment options.

Bacteria are a large group of one-celled organisms. They can live in different places in your body and on your skin. While some types of bacteria are harmless or even beneficial, others can cause infections and disease. A Gram stain helps diagnose harmful bacteria.

Under a Gram stain, different kinds of bacteria change one of two sets of colors (pink to red or purple to blue) under a special series of stains and are categorized as "gram-negative" or "gram-positive," accordingly. Gram staining works by differentiating bacteria by the chemical and physical properties of their cell walls.

However, not all forms of bacteria can be tested using the Gram stain method, and Gram stains don't usually provide a diagnosis alone. Instead, they help to broadly determine the type of bacteria.

Gram staining is an essential staining technique in microbiology that scientists have used for hundreds of years. It's named after Danish bacteriologist Hans Christian Gram, who first introduced it in 1882, mainly to identify organisms causing pneumonia.

What are gram-positive and gram-negative bacteria?

If bacteria are present on a Gram stain slide, a medical laboratory scientist classifies them as gram-negative or gram-positive based on which color they turn under a series of stains. There are also gram-variable bacteria, which means they stain irregularly, and there's a mix of pink- and purple-colored bacteria.

It's important to note that gram-negative doesn't mean there are no bacteria, and "positive" and "negative" don't mean "good" and "bad" in this case.

Gram-positive organisms: Gram-positive bacteria have cell walls that contain thick layers of peptidoglycan, a substance that forms the cell walls of many bacteria. The peptidoglycan forms about 90% of the cell wall in gram-positive bacteria. This causes them to appear blue to purple under a Gram stain.

Gram-positive organisms include:

Staphylococcus species, *Streptococcus* species, *Corynebacterium* species, *Clostridium* sps. *Listeria* sps.

Gram-negative organisms: Gram-negative bacteria have relatively thin or absent peptidoglycan layers.

Neisseria gonorrheae and Neisseria meningitides,Moraxella species, *Escherichia coli* (*E. coli*), *Pseudomonas* species, *Proteus* species, *Klebsiella* species.

Gram Stain Procedure

1. Label your slide. Prepare your smears on a slide with a Gram negative on the left, your unknown in the middle, and a Gram positive on the right.

- Don't forget to methanol fix your slide!

2. Laying your slide on the staining rack, cover the smears with crystal violet for 1 minute.
   - Use just enough stain to fully cover the smear but not so much that it runs or drips off the slide.
3. Tilt the slide and pour the crystal violet off and briefly rinse with water from your wash bottle.
   - Remember not to spray directly on the smears or you will wash them off.
4. Flood the slide with iodine mordant for 1 minute.
   - Tilt the slide, pour off the excess iodine and gently decolorize with Gram's decolorizer until it just begins to run clear. =>This is the tricky part!
5. Tilt your slide on some paper towels to remove excess decolorizer.
6. Flood your slide with the safranin for 1 minute.
7. Tilt the slide to pour off the excess safranin and gently rinse with water until it runs clear.
8. Let the slide air dry
9. Observe under oil immersion.
   - The Gram positive control should be purple and the Gram negative control should be pink.
10. Discard your used slide in the disinfectant bucket.

Draw the gram-positive and gram-negative stained species under high magnification. Also take picture using your mobile under high magnification.

Gram-positive

Gram-negative

What's the difference between a Gram stain and a bacteria culture?

Healthcare providers use both Gram stains and bacteria cultures if they suspect you have a bacterial infection. However, these tests provide different information.

Providers usually request bacteria culture tests alongside Gram stain tests. In a bacteria culture test, a medical laboratory scientist takes cells from the sample that's also for the Gram stain and puts it in a special environment to encourage cell growth. Results are often available within a few days, but some types of bacteria grow slowly, which may take several days or longer. Once the cells have multiplied and grown enough, a medical laboratory scientist examines the cultured bacteria to determine their exact identity.

A Gram stain involves applying a stain to a sample in glass microscope slides and looking at it under a microscope to determine if bacteria are present at all. If bacteria are present, the medical laboratory scientists can see if they're gram-negative or gram-positive based on if the bacteria appear pink to red or purple to blue under the stain. Different types of bacteria are either gram-negative or gram-positive.

While Gram stains provide quicker results than bacteria cultures, in most cases, a Gram stain can't diagnose the type of bacterium, unlike a bacteria culture. However, Gram stains can help point healthcare providers in the right direction for prescribing treatment.

How do I prepare for a Gram stain test?

You don't need to do anything special to prepare for a Gram stain test.

How is a Gram stain test performed?

There are three general steps involved in a Gram stain test, including:

- Collecting the sample.
- Processing the sample.
- Examining the sample.

Collecting the Gram stain sample

A Gram stain test involves your healthcare provider collecting a sample from a site of suspected infection. Some of the ways a provider can collect Gram stain test samples include:

- Brushing or scraping tissue from the surface of a part of your body.
- Collecting fluid or discharge samples from your body.
- Using fine-needle aspiration to draw a fluid sample from an area in your body.

Sites collected by a swab with brushing and scraping may include your:

- Throat, Nostrils, Genitals, Skin wounds.

Samples that you can collect directly into a sterile container include:

- Spit (sputum). Urine (pee).Stool (poop).

Areas in your body that may require the use of fine-needle aspiration include:

- Synovial fluid in your joints. Fluid around your heart (pericardial fluid).
- Fluid around your lungs (pleural fluid). Cerebrospinal fluid (CSF) around your spinal cord.

Your provider then puts the sample in a sterile container and sends it to a laboratory for testing.

Gram staining procedure

At the lab, a medical laboratory scientist smears or spreads the sample on glass microscope slides. These slides are known as smears. They then apply a series of stains to the smear to perform a Gram stain.

The Gram staining process includes four basic steps, including:

1. Applying a primary stain (crystal violet).
2. Adding a mordant (Gram's iodine).
3. Rapid decolorization with ethanol, acetone or a mixture of both.
4. Counterstaining with safranin.

Examining the Gram stain

The medical laboratory scientist then categorizes any bacteria that may be present by color and shape during the microscopic evaluation:

- **Color**: Typically, bacteria that are gram-positive appear purple to blue, and bacteria that are Gram-negative appear pink to red.
- **Shape**: The most common shapes include round (*cocci*) or rod-shaped (*bacilli*).

The medical laboratory scientist also looks for additional characteristics of the sample by observing the groupings of the bacteria on the slide. Examples include:

- *Cocci* that are present singly, in pairs, in groups of four, in clusters or in chains.
- *Bacilli* that are thick, thin, short, long or have enlarged spores on one end.
- If bacteria are present within white blood cells.

The medical laboratory scientist then puts together a report and sends it to your healthcare provider.

What are the risks of a Gram stain test?

There's no risk to having a Gram stain that involves a swab or fluid collection of sputum (spit), urine or stool.

There's very little risk to having blood tests. You may have slight tenderness or a bruise at the site of the blood draw, but this usually resolves quickly.

Possible fine-needle aspiration risks include:

Discomfort or soreness, Bleeding, Infection, Damage to nearby structures.

What do the results of a Gram stain mean?

Gram stain test results reveal one of two categories: a negative Gram stain or a positive Gram stain. This is not to be confused with gram-negative bacteria or gram-positive bacteria.

Negative Gram stain

If your test result reveals a negative Gram stain or "no organism seen," it usually means that there are too few

bacteria present to be able to be seen using the Gram stain method. Bacteria might still be detected by culture if a culture is performed on the specimen.

Positive Gram stain

If your test result reveals a positive Gram stain, it means that bacteria were present in your sample. If your result is positive, it usually includes information about what kind of organism was present on the sample slide, including:

- **Type of bacteria**: Gram-positive or gram-negative.
- **Shape of bacteria**: Round (*cocci*) or rods (*bacilli*).
- **Other bacteria characteristics**: Size, relative quantity (number) and/or arrangement of the bacteria, if applicable.
- **Other cells**: Whether there are bacteria present within other cells (intracellular) and if there's a presence of red blood cells or white blood cells.
- **Fungi**: Gram stains can check for the presence of fungi in the form of yeasts or molds. You may need further testing to identify the specific type.

This information, along with signs and symptoms and other clinical findings, will help your healthcare provider determine which treatment may be most effective, sometimes before bacteria culture results are available.

When should I know the results of the test?

Gram stains generally provide quick results. However, Gram stain results are usually considered preliminary, meaning they can't always provide a diagnosis alone. Results of a bacteria culture and/or other tests, such as antigen and antibody testing for particular types of bacteria, might be necessary to confirm a diagnosis.

When should I call my doctor?

If you've been diagnosed with an infection and are having issues with your treatment or your infection isn't improving, contact your healthcare provider.

A note from Cleveland Clinic

Gram stains are a very common and often painless way to determine the presence of a bacterial or fungal infection. While it can be stressful to wait for the results of a diagnostic test, know that your healthcare team is there to support you no matter what the results are. Don't be afraid to ask your healthcare provider questions about your test or results.

Give a few examples of gram-positive bacteria.

Which is more harmful- gram-positive bacteria or gram-negative bacteria?

Is it easier to kill gram-positive bacteria?

What infections are caused by gram-positive bacteria?

Which infections are caused by gram-negative bacteria?

Chapter 9: Acid-Fast Stain

Objectives:

- Describe the difference between acid-fast bacteria and non-acid-fast bacteria.
- Explain how the acid-fast stain works comparing acid-fast and non-acid fast bacteria.
- Identify the genera of bacteria that are acid-fast and two examples of diseases caused by these species.
- Differentiate the cell wall structures of acid-fast and non-acid fast bacteria.
- Successfully execute an acid-fast stain and interpret the results.

Introduction:

Acid Fast Stain

Acid fast stain is a differential stain used to identify acid-fast organisms such as members of the genus *Mycobacterium*. Acid-fast microorganisms are characterized by wax-like, nearly impermeable cell walls; they contain mycolic acid and large amounts of fatty acids, waxes, and complex lipids. This type of cell wall is resistant to most compounds, therefore acid-fast microorganisms require a special staining technique.

The ability of the bacteria to resist decolorization with acid-alcohol confers acid-fastness to the bacterium. Acid-fast bacteria, of which there are very few---the major genus *Mycobacterium*, have a high concentration of mycolic acid, a lipid, in their cell walls. Although difficult to stain, once the stain goes into the cell wall, the cell will not de-stain or decolorize easily. The ability of the bacteria to resist decolorization with acid-alcohol confers acid-fastness to the bacterium. The phenol in the carbol fuchsin facilitates the dye going into the waxy wall of the bacterium. Acid-fast bacteria stain poorly with the Gram stain procedure, appearing weakly Gram-positive or Gram-variable. They are usually characterized using the acid-fast staining procedure.

Steam is used to get the carbol fuchsin primary dye to go into the cell wall. Once in, it will not come out: But the acid-alcohol decolorizer will take it out of the non-acid fast cell walls since the primary dye does not bind strongly to the cell wall. Non-acid fast bacteria will also take up the carbol fuchsin, but the acid alcohol decolorizer will remove it from wall since the primary dye does not bind strongly to the cell wall.

The primary stain used in acid fast staining, carbol fuchsin, is lipid-soluble and contains phenol, which helps the stain penetrate the cell wall. This is further assisted by the addition of heat in the form of heat (steam). Steam helps to loosen up the waxy layer and promotes entry of the primary stain inside the cell. The smear is then rinsed with a very strong decolorizer (acid-alcohol), which strips the stain from all non-acid-fast cells but does not permeate the cell wall of acid-fast organisms. The decolorized non-acid-fast cells then take up the counterstain, which in our case is methylene blue.

Acid-fastness is an uncommon characteristic shared by the genera *Mycobacterium* and *Nocardia* (weakly acid-fast). Because of this feature, this stain is extremely helpful in identification in diseases caused by acid-fast bacteria, particularly tuberculosis and leprosy. In addition, the stain is used to determine the presence of acid-fast bacteria from lung tissue in patients undergoing antibiotic therapy.

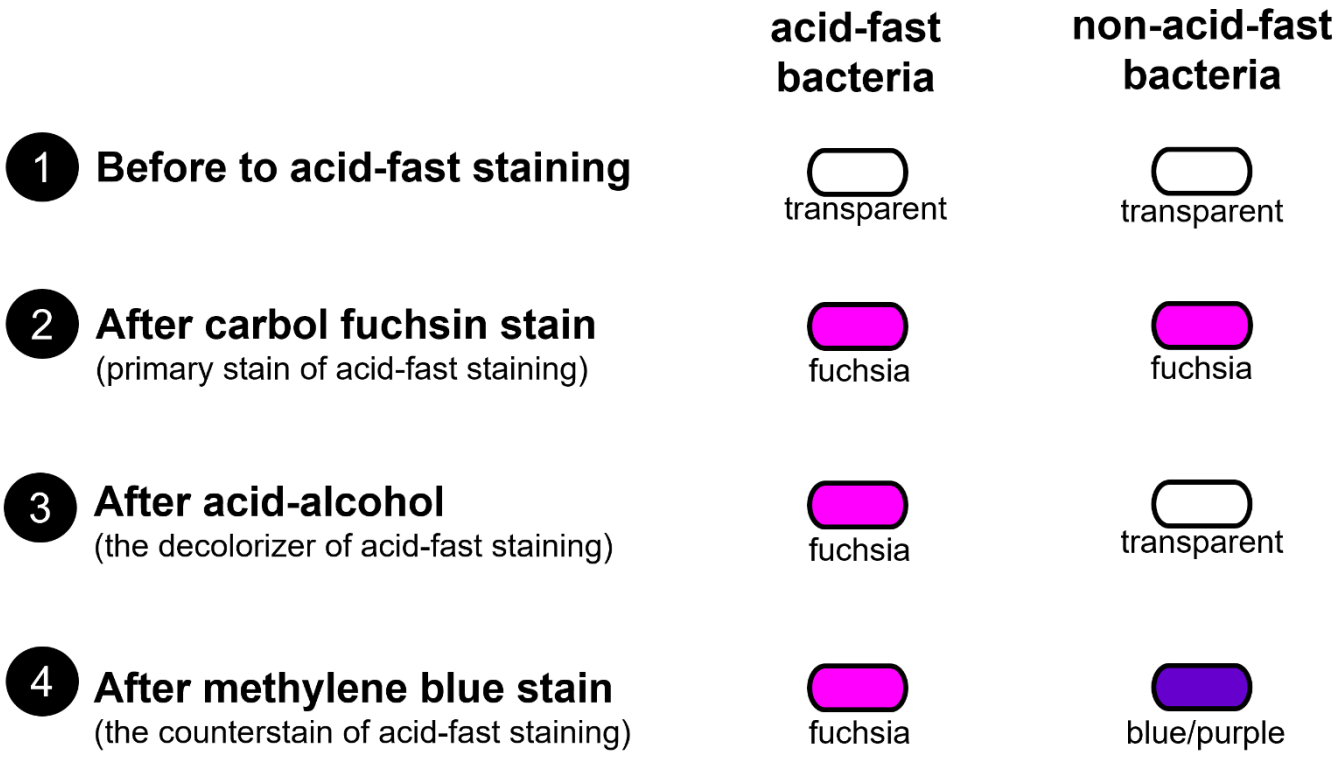


Figure 9. 1: Diagram shows how cells change color during the steps of the acid-fast stain by comparing acid-fast bacteria and non-acid-fast bacteria. Acid-fast bacteria and non-acid-fast bacteria begin transparent. After staining with carbol fuchsin, acid-fast and non-acid-fast bacteria both appear the color fuchsia (a pinky-purple color). After the decolorization step, acid-fast bacteria retains the carbol fuchsin and remains fuchsia and the non-acid fast bacteria looses the color from the carbol fuchsin becoming mainly transparent. Methylene blue counterstain causes non-acid-fast bacteria to stain blue or bluish-purple, whereas acid-fast bacteria remain fuchsia.

Structure and Composition of the Acid-Fast Cell Wall

The mycobacterial cell wall resembles both the Gram-positive and Gram-negative cell walls by having a peptidoglycan layer nearly as thick as Gram-positive cell walls and an outer, waxy layer mimicking the outer membrane of Gram negative cell walls. The cell wall of mycobacteria plays a key role in intrinsic antibiotic resistance and virulence as described by Forrellad *et al*. 2013; and Becker and Sander 2016.

Acid-fast bacteria are Gram-positive, but in addition to peptidoglycan, the outer membrane or envelope of the acid-fast cell wall of contains large amounts of glycolipids, especially mycolic acids that make up approximately 60% of the acid fast cell wall in the genus *Mycobacterium*.

The following is a list of the layers of the acid-fast cell wall beginning with the innermost layer (nearest to the plasma membrane):

- **Layer 1:** Layer of peptidoglycan
- **Layer 2:** Layer of arabinogalactan (a long carbohydrate composed of the sugars arabinose and galactose)
- **Layer 3:** Outer membrane containing mycolic acids
- **Layer 4:** Outer surface is studded with surface proteins that differ with the strain and species of the bacterium

Like the outer membrane of the Gram negative cell wall, porins are required to transport small hydrophilic molecules through the outer membrane of the acid-fast cell wall.

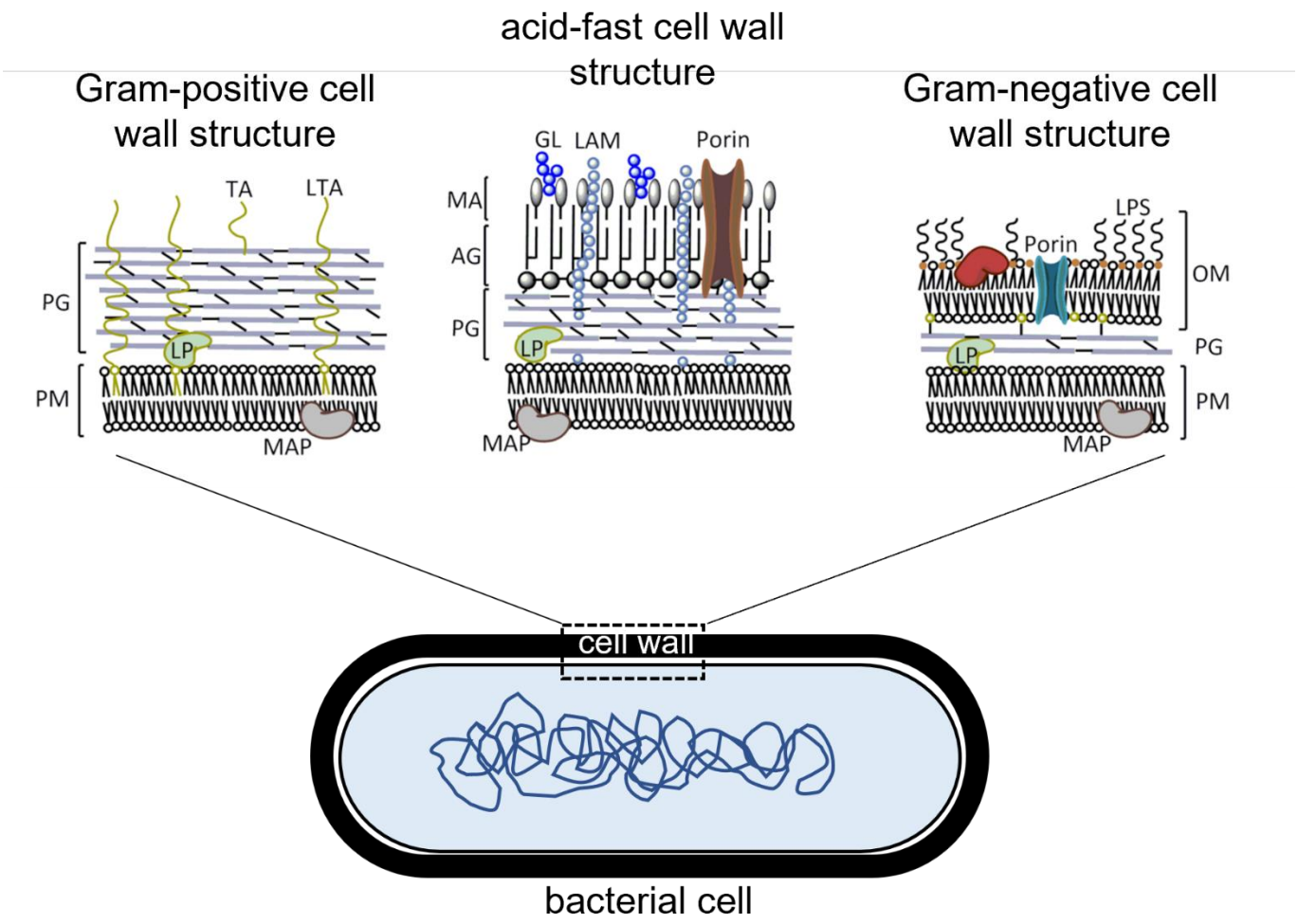


Figure 9.2: Comparison of the structures of different types of bacterial cell walls. (Left) Gram-positive cell walls consist of a thick layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM) with teichoic acid (TA) and lipoteichoic acid embedded across the peptidoglycan layer. (Right) Gram-negative cell walls consist of a thin layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM). Gram-negative cell walls have an outer membrane (OM) beyond the peptidoglycan layer containing lipopolysaccharide (LPS) as well as porin channels that enable some materials to pass across the outer membrane. (Center) Acid-fast cell

walls have a moderately thick layer of peptidoglycan (PG) outside of the plasma membrane. A layer of arabinogalactin (AG) is outside of the peptidoglycan layer and beyond the AG layer is mycolic acid (MA) containing lipoarabinomannan (LAM) and glycolipid (GL). In all three cell wall types, lipoprotein (LP) is also found.

1. Label a slide and draw a circle on the center of the slide with a wax pencil.
2. Prepare an emulsion on the slide with 4 loopfuls of *Staphylococcus epidermidis* from a broth culture onto the slide (these will be your non-acid-fast bacteria).
3. Then, add one loopful of *Mycobacterium chelonae* (these are your acid-fast bacteria) and mix the two bacteria together.
4. Allow the slide(s) to air dry on the slide warmers (while these slides are drying).
5. Once the liquid has completely evaporated, heat fix the bacteria by passing it through your flame three times.
6. Make sure the slide rack on top of your beaker is completely level. Then, bring your water to boil while the slides are drying. You only need about 200 milliliters of water. If you add more, you will be waiting all lab period for your water to boil.
7. Once the water is boiling, place your slide on the slide rack above the boiling water.
8. Cover the area of your smear on the slide with a square piece of paper towel. Cut the paper towel to make sure none of the paper is hanging off the slide.
9. Carefully apply the carbol fuchsin stain to the paper towel. If a stain appears in the water you are boiling, please stop and discard the stained water in the liquid waste disposal. The fumes from carbol fuchsin can be toxic.
10. Steam with the stain on the slide for 7 minutes while continuously applying more stain so the paper square never dries out.
11. Gently remove the paper with forceps and discard it in the small waste paper cup that will be provided on your bench. Then, rinse the slide with water.
12. Put the slide over the staining basin and gently rinse with water.
13. Decolorize with 6 drops of acid-alcohol (not ethanol from the Gram stain kit), then rinse with water.
14. Counterstain with methylene blue for 2 minutes.

15. Rinse with water and blot dry with bibulous paper (do not use the slide warmer).

16. Examine under the 100x objective lens with oil immersion and record your results.

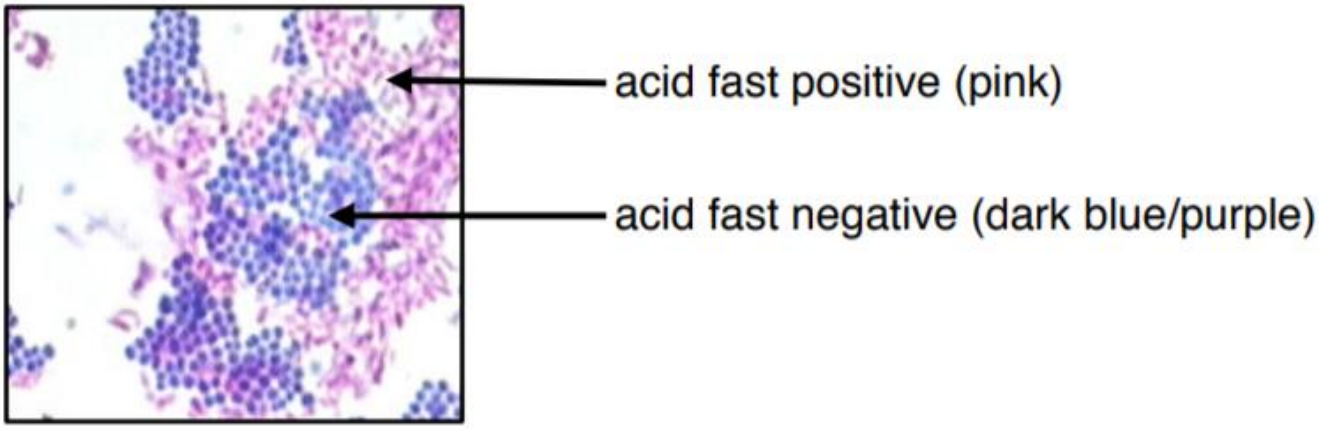


Figure 9.3: Microscopic image of two different species mixed together with acid fast stain. The acid-fast bacteria appears fuchsia or pinky-purple. The non-acid-fast bacteria appears dark blue or blue-purple. The colors in this image may be slightly off due to printing/copying.

Questions

1. Take a photo of the cells or make an illustration of them.

2.

3. Was your acid-fast stain successful? How do you know?

4. What color are the acid-fast bacteria?

5. What color are non-acid-fast negative bacteria?

6. Describe the structure of the cell walls of bacteria that appear fuchsia with the acid-fast stain.

7. What is the medical importance of using the acid-fast stain (what diseases/infections can it help to identify and how might it relate to virulence and antibiotic resistance)?

Chapter 10: Congo red Capsule Stain

Objectives:

- Describe what bacterial capsules are and where they are found in bacterial cells.
- Tell how the capsule stain works.
- Give at least three ways bacterial capsules benefit bacterial cells.
- Successfully conduct a capsule stain.
- Examine, illustrate, label, and interpret results of a capsule stain.

Introduction

The procedure for staining for capsules is different than the procedure used for most other stains. The Congo Red Capsule stain is a modification of the nigrosin negative stain you may have done previously. The bacteria take up the congo red dye and the background is stained then with acid fuchsin dye. The capsule or slime layers, highly hydrated polymers, exclude both dyes. The background will appear blue, the bacterial cells will appear pink, and the clear halos are the capsules. Bacterial capsules are typically composed of a polysaccharide layer which is thick, detectable, and discrete layer outside the cell wall Figure 10.1). Capsules do not stain well for microscopic examination. Because of this, to visualize bacterial capsules, stains/reagents are used in a way that will stain the environment surrounding the bacterial cells and stains the bacterial cell itself, but not the capsule. This creates a non-stained area that is the capsule. Capsules appear as uncolored halos surrounding the bacterial cell.

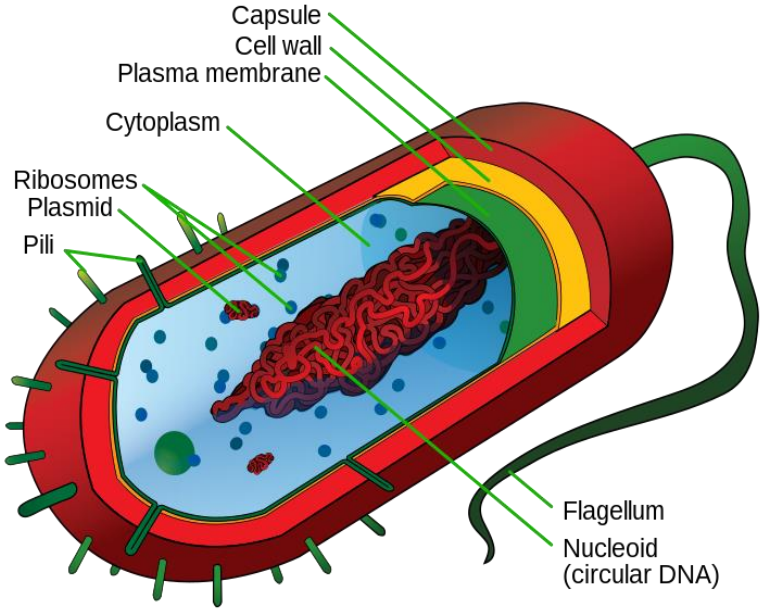


Figure 10.1: Diagram of a prokaryotic cell shows that capsules are a layer located outside of the cell wall.

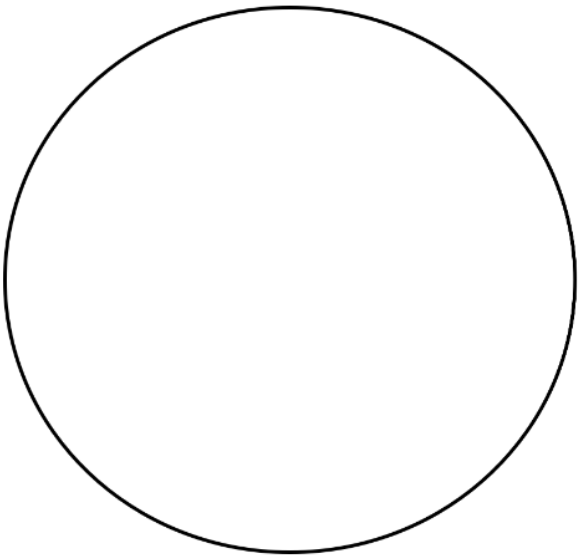
Clinically, the capsules of some highly pathogenic bacteria (i.e.: pneumococci, *Haemophilis influenzae*, and meningococci), can be distinguished with the use of antisera specific for that type of capsule. The bacteria are suspended in the antisera and then mixed with methylene blue. In the antisera staining procedure, the bacteria will appear blue surrounded by a clear halo and then surrounded by a thin blue line where the antisera have attached to the capsule

The ability to produce a capsule is coded in the DNA of the bacteria and can therefore be species-dependent or even strain-dependent. The capsule is not an absolutely essential cellular component for bacteria and some species and strains do not form capsules. Capsules are often produced only under specific growth conditions. The thickness of the capsule can vary depending on the bacterial species, its age, and the medium in which the bacterium is growing. Even though not essential for life, capsules can help bacteria to survive.

Capsules protect pathogenic bacteria from the phagocytic action of immune cells and allow pathogens to invade the body by enabling them to avoid being engulfed by white blood cells. If a pathogenic bacterium loses its ability to form capsules, it often ceases to be pathogenic. Some bacterial species of the microbiome also have capsules to protect them from phagocytosis. Environmental bacteria living in soil and water are protected by their capsules from being engulfed by free-living protozoa.

In addition to protection from phagocytosis, the capsule protects cells against desiccation (drying out). Capsules also enhances the ability of cells to attach. For example, pathogenic bacterial cells with capsules aid in attachment to host cells to establish their growth/infection. Further, capsules enable bacterial cells can attach to other bacteria in biofilms. Bacteria that establish the first layer of a biofilm may utilize their capsules to adhere to a surface and enable attachment of other bacterial cells for biofilm development.

Illustrate the bacterial cells viewed in the microscope.



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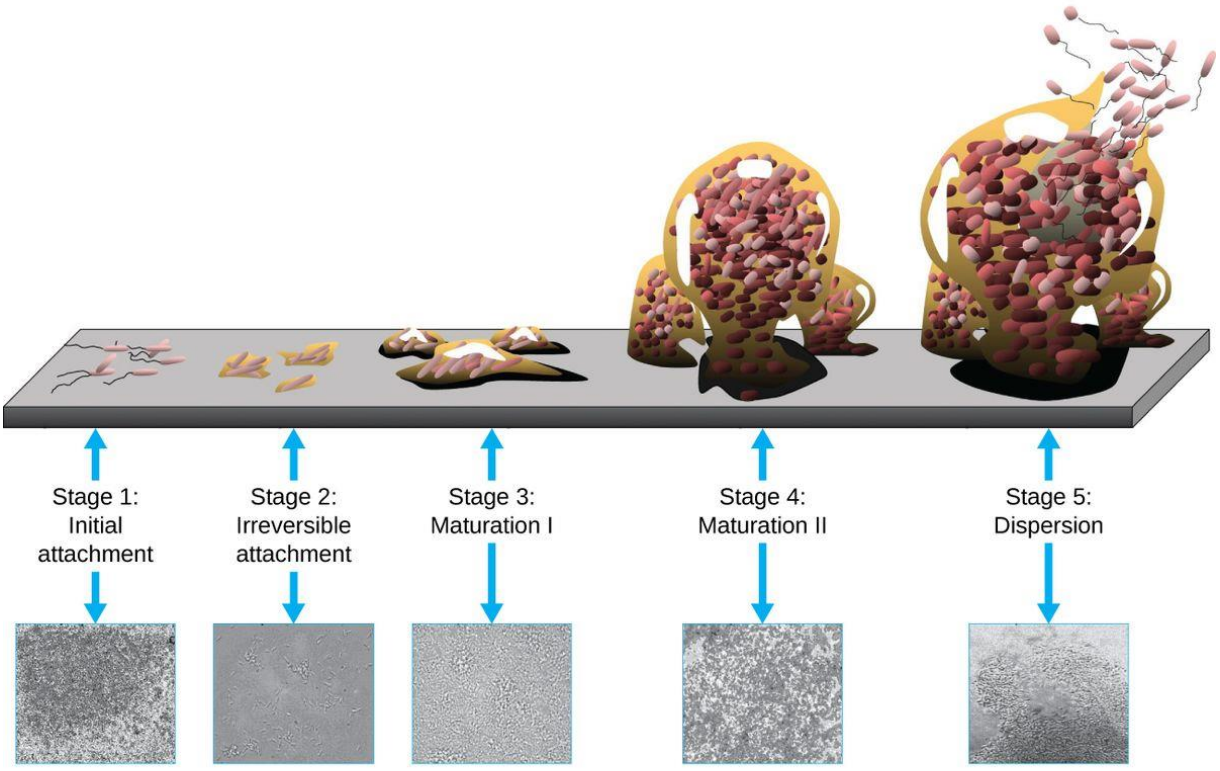


Diagram showing five stages of biofilm development of *Pseudomonas aeruginosa*.
All photomicrographs are shown to same scale.

Figure 10.2: Process of biofilm development. Stage 1: initial attachment of bacteria to a surface. Stage 2: irreversible attachment. Stage 3: maturation I. Stage 4: maturation II. Stage 5: dispersion of cells out of the biofilm.

Materials: Congo Red stain, Acid fuchsin stain, Acid alcohol, *Klebsiella pneumoniae*, *Enterobacter aerogenes*

Procedure:

- Place a loop-full of Congo Red on a slide
- Mix a small amount of your organism into the drop of Congo Red.
- Spread the organism/dye suspension well on the slide
- Let the slide thoroughly air dry. Do not methanol fix!
- Fix the dried slide with acid alcohol for 15 seconds.
- Rinse with distilled water and cover the slide with acid fuchsin for 1-5 minutes.
- Rinse with water and allow to air dry.
- Examine the slide under oil immersion. Cells stain red/pink, and the capsules appear as colorless halos against a dark blue background.

Capsule Stain (Anthony's Capsule Stain), OPTIONAL PROCEDURE

1. **Gently** stir the skim milk broth culture with your loop and place **2-3 loops** of *Enterobacter aerogenes* or *Serratia marcescens* (whichever is available) on a microscope slide.
2. Using your inoculating loop, spread the sample out to cover about one inch of the slide.
3. Let it completely air dry. ***Do not heat fix or use the slide warmer***. Capsules stick well to glass, and heat may destroy the capsule.
4. Stain with 1% crystal violet for two minutes (***do not use the same crystal violet solution as the Gram stain***).
5. ***Gently*** wash off the excess crystal violet stain with 20% copper sulfate solution.
6. Gently shake off the excess copper sulfate solution and let air dry (***do not blot or use slide warmer***).
7. Examine the slide with the microscope. The bacterial cell and the milk dried on the slide will pick up the purple dye while the capsule will remain colorless.

Questions

What type of molecule are bacterial capsules typically composed of?

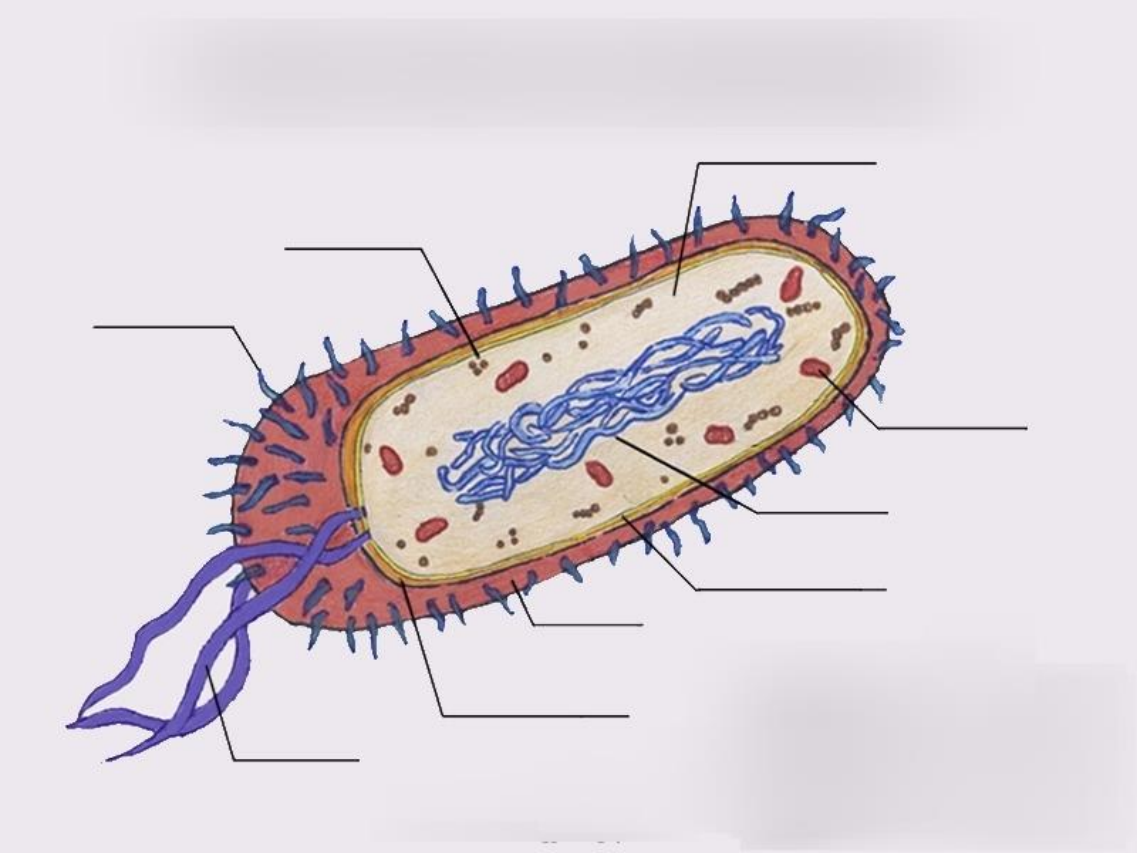
Where are bacterial capsules located in a bacterial cell?

Why do bacterial capsules appear white/clear after staining?

Give three different ways bacterial capsules give species/strains that have them an advantage.

Do all bacteria have/need a capsule? Explain your answer.

Label a bacterial cell and a capsule in your illustration.



Chapter 11: Endospore Stain

Objectives

- To describe what an endospore/spore is and why they are important for the bacterial species that form them.
- To identify/give examples of environmental conditions that can stimulate spore formation.
- To tell that *Bacillus* species and *Clostridium* species can be clinically important endospore-forming species.
- To compare and contrast "vegetative cell" and "spore." And Successfully conduct an endospore stain.
- To identify when endospores are terminal, subterminal, and central in microscopic images, diagrams, and descriptions.
- To tell how the endospore stain works including the stains involved and how the stains penetrate cells and do or do not wash out of cells.
- To apply the concept of bacterial endospores to healthcare settings and how spores can make treatment of infections by spore-forming species challenging.

Introduction

Endospore is a form of a bacterial cell. Endospores are made so the cell can survive poor growth or poor survival conditions. Only some species of bacteria can produce endospores. An endospore is a mainly inactive version of a cell that is analagous to a survival bunker. When environmental conditions are poor, or even deadly, bacteria that are able to will produce endospores to survive. When environmental conditions improve, endospores can produce the active cells again (active cells are called **vegetative cells**.) Bacterial endospores are the most resistant structures of all living organisms, and they can live in this dormant dehydrated state for hundreds of years (even some documented at thousands of years). The stimulus that triggers sporulation (formatio of spores) can vary and may include nutrient depletion, desiccation, chemicals, heat, etc. Endospores are not for reproduction. One spore forms inside of one vegetative cell (vegetative = metabolically active cell). When environmental conditions improve, one spore germinates to produce one vegetative cell.

Endospore production is a very important characteristic of some bacteria, allowing them to resist adverse environmental conditions such as desiccation, chemical exposure, extreme heat, etc. Endospores were first identified in the 1800s (John Tyndall developed a process for destroying them with an intermittent heating procedure), although the stain procedures to identify them did not develop until the early twentieth century.

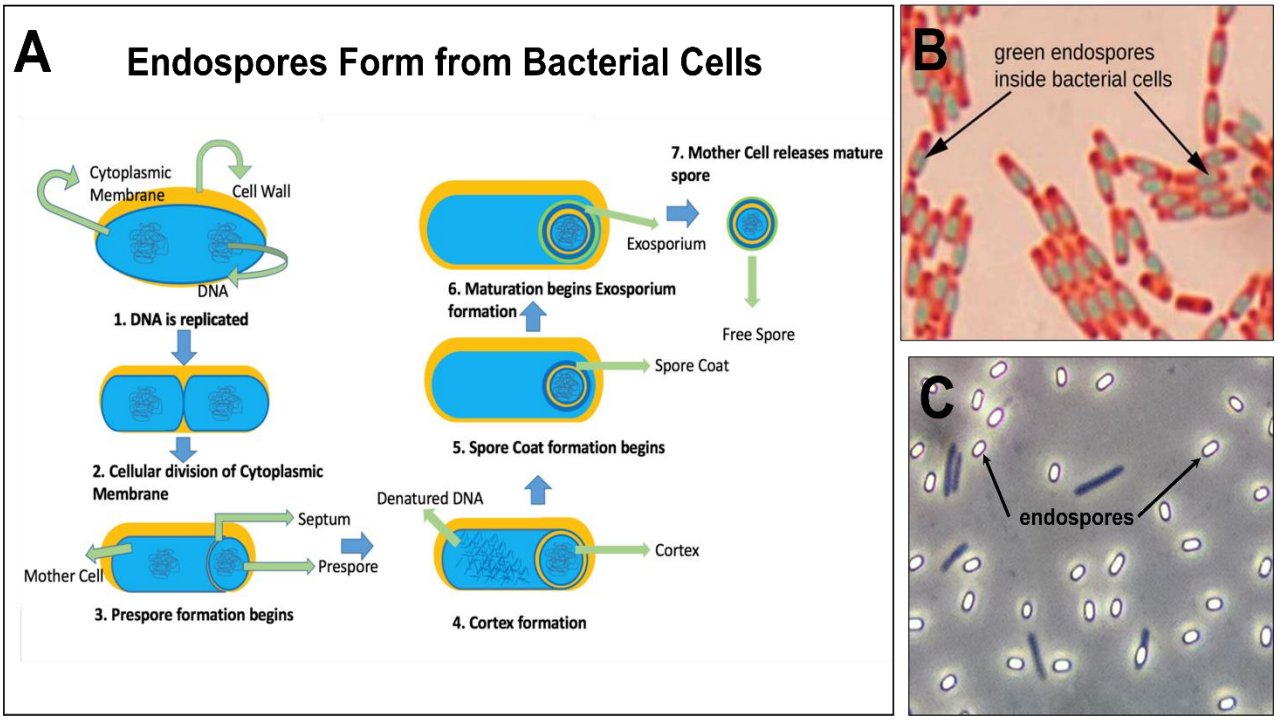


Figure 11.1: (A) Endospores form from bacterial cells. Diagram shows the process how bacterial cells experiencing poor growth conditions can form endospores in the following steps: 1. DNA is replicated, 2. cellular division of cytoplasmic membrane, 3. prespore formation begins, 4. cortex forms, 5. spore coat formation begins, 6. maturation of exosporium formation, 7. mother cell releases mature spore. (B) Microscopic image prepared with an endospore stain shows bacterial cells (pink) with forming spore inside of them (green). (C) Microscopic image showing endospores.

The identification of endospores is very important for the clinical microbiologist who is analyzing a patient's body fluid or tissue since there are not that many spore-forming genera. Knowing if the species of bacteria causing an infection forms spores or not helps to narrow down the possible bacterial species causing the infection. In fact, there are two major pathogenic (disease-causing) spore-forming genera: *Bacillus* and *Clostridium*. *Bacillus* and *Clostridium* species cause a number of dangerous and lethal diseases such as botulism, gangrene, tetanus, c-diff, and anthrax, to name a few.

Some bacteria have to be put into unfavorable situations (high cell density and starvation are two key triggers) to go into sporulation. Other species will make spores easily without much provocation (e.g. *Bacillus subtilis*). Vegetative cells that have not yet made spores may be in the process of making the spore or will not make them at all. The vegetative cell is metabolically active, whereas the spore is not. The location where an endospore is within a vegetative cell is also useful for distinguishing bacterial species. Endospores may be located in a terminal (end of the cell), subterminal (near the end of the cell), or central (middle of the cell) position. A particular species of the genus will form spores in a specific area,

producing another useful taxonomic identification tool and therefore useful in identifying the species of bacteria.

Endospore Stain

The endospore stain is a differential stain that enables visualization of endospores and differentiation of spores from vegetative cells. The primary stain, malachite green, is a relatively weakly binding stain that attaches to the cell wall of vegetative cells and the spore wall of endospores and mature spores. In fact, if washed well with water, the stain comes right out of the cell walls of vegetative cells. In contrast, malachite green will not wash from the spore wall. Once the stain is in the spore wall, it is locked in and will not wash out. This is why there does not need to be a decolorizer in this stain procedure.

It is difficult to stain the tough spores. Heat is used to enable the malachite green to penetrate the low-permeability spore walls. A variety of chemicals make up the spore wall (keratin protein, calcium), but deeper in the wall is peptidoglycan. The keratin forming the outer portion of the endospore wall resists stain. Heating the cells will make the spore wall more permeable to the malachite green so it can then attach to the peptidoglycan. Once in, the malachite green will not come out because the overlying spore wall becomes less permeable when the smear cools.

When the malachite green is washed out of the vegetative cells they become transparent. A counterstain, safranin, is used to stain the vegetative cells pink. The result is endospores that appear green and vegetative cells that appear pink.

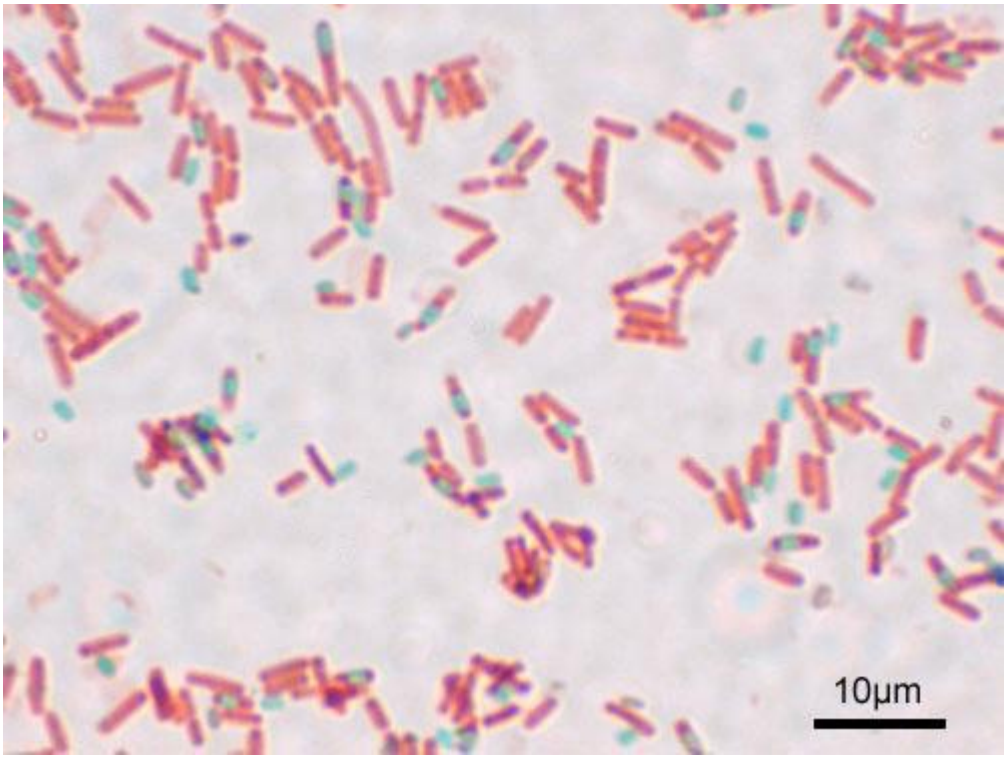


Figure 11.2: Bacterial cells that have undergone the endospore stain. This species, *Bacillus subtilis*, forms endospores that are visible in green. The vegetative cells are also present and appear pink.

Prepare a Bacterial Smear

You may find it helpful to draw a circle (wax pencil is best) on the opposite side of the slide where you will spread your smear. This will help you later in locating the smear. The wax pencil is better than a marker because it will not wash off easily from the glass. If you are using a liquid culture, gently mix the culture until you get an even, cloudy mixture (it should look somewhat like skim milk). If you mix too aggressively, you will lose the bacterial morphology.

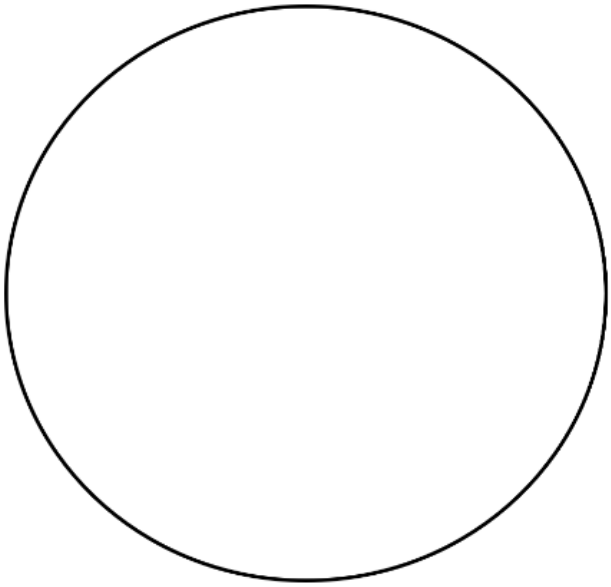
1. Prepare a bacterial smear on the slide:
    - If you are taking bacteria from a solid culture (slant or petri plate), place a small drop (only 1 drop) of saline, deionized (DI) water, or distilled water onto a microscope slide and use a loop to aseptically add bacteria to the water (avoid taking a large chunk of bacteria since the cells will be too dense to view individual cells). Use the loop to spread the bacteria in the water drop and to spread the water drop out to make it thinner (it will dry faster).
    - If you are taking a bacteria from a a liquid culture (broth), place 1 or 2 loopfuls of bacteria directly onto a microscope slide (no saline or water added).
2. Allow the slide(s) to air dry on the slide warmer (or air dry if a slide warmer is not available)

If you heat fix too little, the bacteria will wash off the slide. If you heat fix too much, you will cook the bacteria and denature them.

Endospore Stain

1. Put a beaker of water on the hot plate and boil until steam is coming up from the water.
2. Turn the hot plate heat down so that the water is barely boiling.
3. Place a wire stain rack over the beaker. Steam should be coming up through the wire rack.
4. Cut a small piece of paper towel and place it on top of the smear on the slide. The towel will keep the dye from evaporating too quickly, thereby giving more contact time between the dye and the bacterial walls.
5. Flood the smear with the primary dye, malachite green, and leave for 5 minutes. Keep the paper towel moist with the malachite green. DO NOT let the dye dry on the towel.
6. Remove and discard the small paper towel piece.
7. Wash the slide really well with water.
8. Move the slide off the wire rack. The next steps are done without the steam of the hot water.
9. Use a wooden clip and attach it to the slide and put the slide over a small chemical waste bucket.
10. Apply safranin to the smear and leave for 1 minute.
11. Wash the slide well with water allowing the water to run off the slide into the bucket.
12. Blot the dry (do not wipe) with bibulous paper.
13. Place the stained slide on a microscope and examine. Be sure to begin at the lowest power (look for the pink and green colors of the stains). Focus at lowest power and then increase one objective

magnification at a time.



specimen: _____

total magnification: _____

1. Draw the cells you observe after the endospore in the space above.

2. What color are the endospores/spores in the endospore stain?

3. What color are the vegetative cells in the endospore stain?

4. Why might a species of bacteria form endospores? What is the advantage of forming spores for bacteria?

5. What genera of bacteria are known to have pathogenic (disease-causing) species and are endospore-forming genera?

6. Why might infections with species of bacteria that produce spores be more difficult to treat in a healthcare setting than species of bacteria that do not produce spores?

7. What is the purpose of the steam in the endospore stain?

8. Why does there not have to be a decolorizer in this stain?

Chapter 12: Blood Agar

Objectives

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, biochemical testing, and media types when making observations.
- Correctly perform various inoculation techniques and describe each technique's purpose.
- Make accurate observations and appropriate interpretations of biochemical test results and use them in the identification of potentially disease causing microbes.

Introduction

Blood Agar Blood agar is used for isolation and cultivation of many types of fastidious bacteria**.** Blood agar is a general purpose, enriched medium often used to grow fastidious organisms and to differentiate bacteria based on their hemolytic properties. Blood agar is usually prepared from tryptic soy agar or Columbia agar base with 5% sheep blood. Blood agar medium that is both enriched and differential. Many bacteria can grow in this medium. Some bacteria can grow on it simply whereas some others can only grow partially or completely break down the blood cells embedded in the agar.

Hemolysis, the ability of an organism to lyse red blood cells and breakdown hemoglobin can be seen when sheep's blood is added to the medium. Substances produced and released (exotoxins) which accomplish this are called **hemolysins**. The addition of blood has a two-fold purpose. It is an **enrichment** to support the growth of fastidious organisms and it is the substrate with which hemolysins, if present, will interact. In blood agar, the hemolytic activity can be visualized without an indicator substance usually added to most differential media.

Before inoculation, a **blood agar** plate appears red and opaque due to the large particulate red blood cells (RBCs) contained within. If an organism growing on the plate produces **beta (β) hemolysins**, the RBCs are completely destroyed or lysed and the medium surrounding the growth loses its opacity. If this is the case, the medium may become transparent enough that you may be able to read printing through the clearing.

If the blood cell membranes are partially lysed by the exotoxin, the RBC contents will leak out without the complete destruction of the blood cell. The hemoglobin from the RBC will be reduced to methemoglobin resulting in a green or brown discoloration to the medium surrounding the colony. This incomplete

hemolysis is called **alpha (α) hemolysis.**"On prolonged incubation, many alpha hemolytic organisms will begin to appear more clear, but if the surrounding medium contains any shades of brown or green the 'hemolysis' is still considered 'alpha.'"

If the bacterium does not produce a hemolytic exotoxin, there will be no change to the RBCs, and the medium will remain opaque red. This lack of hemolysis is classified as **gamma (ϒ) hemolysis**.

Hemolysins are involved in the pathogenicity of bacteria. These membrane-disrupting **exotoxins** affect cell membrane function either by forming pores or by disrupting the phospholipid bilayer in host red blood cell membranes. They cause leakage of the cytoplasmic contents and cell lysis. They can affect other cells as well. The gram-positive bacterium *Streptococcus pyogenes* produces two types of streptolysins, O and S. Streptomycin O is not active in the presence of oxygen (**oxygen labile**), whereas streptolysin S is active in the presence of oxygen (**oxygen stable**). Other important pore- forming membrane-disrupting toxins include alpha toxin of *Staphylococcus aureus* and pneumolysin of *Streptococcus pneumoniae*.

Blood Agar (TSB w/ 5%SB) Pancreatic digest of casein 14.5 g/L Peptic digest of soybean meal 5.0 g/L Sodium chloride 5.0 g/L, Agar 14.0 g/L, Defibrinated Sheep Blood 5.0%

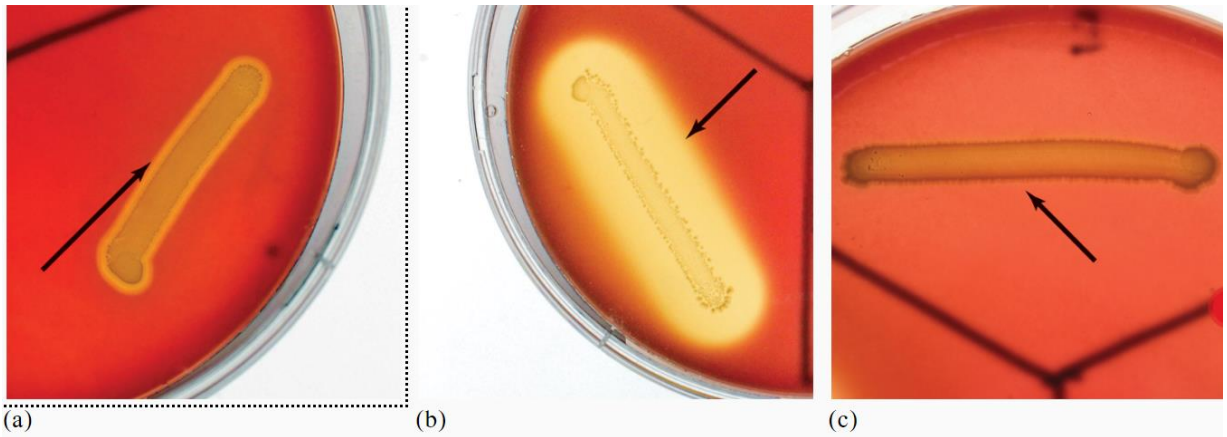


Figure 12.1: Alpha-hemolysis (a), beta- hemolysis (b); gamma- hemolysis (c)

Material

Streptococcus pyogenes

Streptococcus sanguinis (sanguis)

Streptococcus (Enterococus) faecalis

Patient A, (Your cheek cells or swap) for diagnosis

1 blood agar plate (TSA w/ 5% SB**)**

Procedure

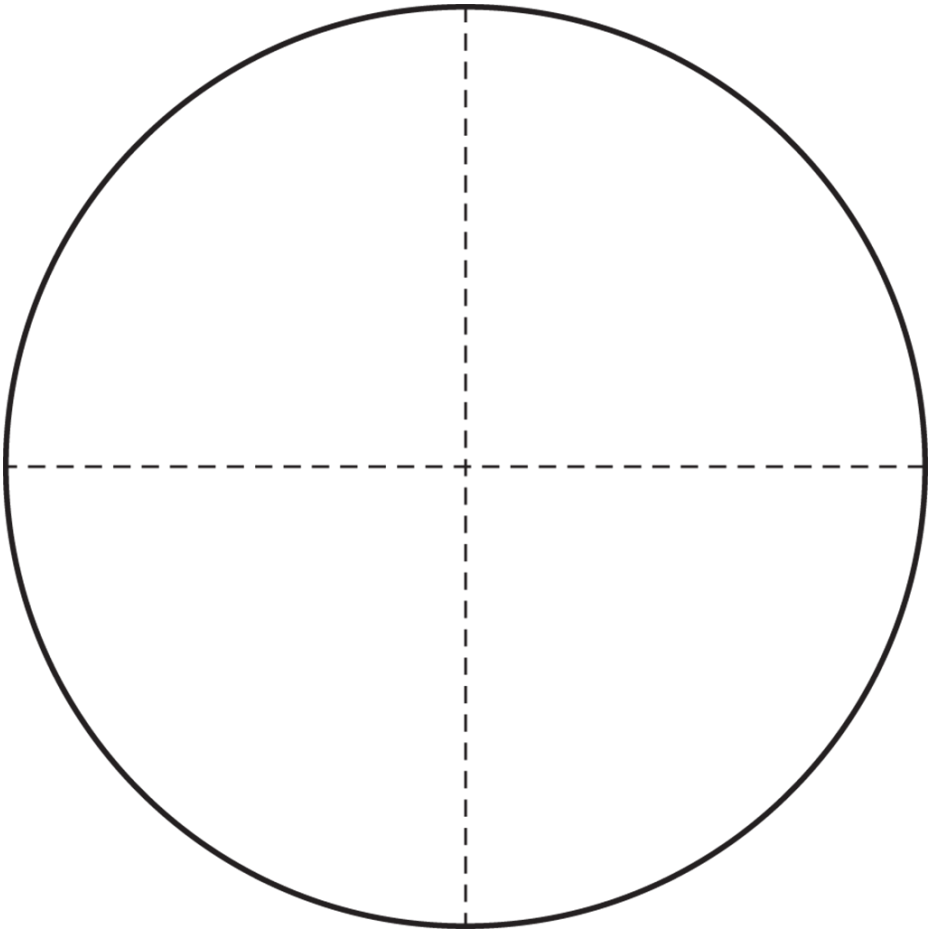
- Obtain one blood agar plate
- Add the following to the bottom of the plate around the edge: your name, date.
- On the bottom, divide each plate into 4 sections. Label each section with an abbreviation for each

organism. Write small but legibly! Each plate will be inoculated with each of the 4 organisms.

- Aseptically make a short (1 cm) streak-stab inoculation of each organism in the corresponding section.
- After obtaining cells on the loop, begin the surface streak at point 1 and ending with a stab at point
- Inoculate each organism into its area with a short straight line/spot inoculation followed by a stab at the end of the streak line.
- Keep the inoculation lines short and away from other inoculations on the plate.
- Be sure to make each inoculation separately and refrain from "double dipping."
- Be sure to hold the lid of the plate above the plate surface to protect it from airborne contaminants.
- It may help to set the plate on a piece of white scratch paper so that you can see the sector lines.
- Place the plates upside-down in the location designated for cultures to be incubated.
- They will be incubated for 24-48 hours at 37°C.

Figure 12.2: Inoculating and reading the blood agar plate.

Draw your result on the circle and photograph with your mobile device.



Did you observe colonies that showed alpha, beta, or gamma hemolysis? Explain why yes and why NOT?

Chapter 13 Mannitol Salt Agar

Objectives:

- Define selective medium and differential medium.
- Explain how mannitol salts agar is selective.
- Explain how mannitol salts agar is differential.
- Successfully utilize mannitol salts agar to characterize/identify bacterial species and interpret results.

Introduction:

Mannitol salts agar is a microbiological medium that is both selective and differential. Selective means the medium will only allow certain microorganisms to grow. Differential means the medium will show some characteristic of the microorganisms growing on it and can be used to differentiate between different species.

Mannitol salts agar is selective since it has a high salt concentration and will only allow halophilic (salt-loving species) or halotolerant (salt-tolerant) species to grow on it. This medium contains 7.5% NaCl (salt), whereas typical media contains about 0.5% NaCl. Species that grow on mannitol salts agar: mannitol utilization and salt resistance positive. Species that do not grow on mannitol salts agar: mannitol utilization/salt resistance negative

Mannitol salts agar is differential since it will detect acid production as a result of fermentation of mannitol (a sugar in the medium) in species that can ferment mannitol to produce acid. The medium contains a pH indicator, phenol red, that is red-orange when the pH is neutral (around pH 7). If a species ferments mannitol and produces acid, the pH of the medium decreases and phenol red will become yellow.

Medium remains red-orange after growth and growth observed: mannitol fermentation negative. Medium is yellow after growth: mannitol fermentation positive. Use of mannitol salts agar is useful for differentiation of species of *Staphylococcus* and *Micrococcus*. Mannitol fermentation by pathogenic staphylococci, such as *Staphylococcus aureus*, is indicated by the media changing to yellow, whereas *Staphylococcus epidermidis*, a non-pathogenic species of staphylococci, does not produce a yellow color.

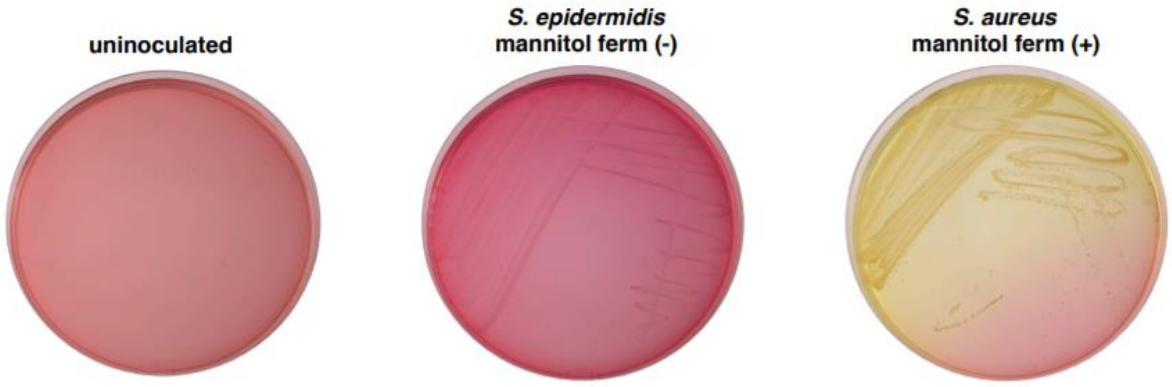


Figure 13.1: Mannitol salts agar. (Left) The uninoculated medium has a light red to red-orange color. (Middle) The medium with *Staphylococcus epidermidis* growing on it shows the appearance with a mannitol fermentation negative species and appears red to red-orange. (Right). The medium with *Staphylococcus aureus* growing on it shows the appearance with a mannitol fermentation positive species and appears yellow in the region of the medium where the bacteria is growing.

Procedure

1. Obtain a mannitol salts agar petri plate and label with your name/group name or number and "mannitol salts agar."
2. Draw a straight line on the underside of the petri plate to separate the plate into two halves. Label one side *Staphylococcus aureus* and the other side *Staphylococcus epidermidis*.
3. Aseptically inoculate each half of the plate with the corresponding bacterial species with a streak that is a straight line in the center of each half of the petri plate.
4. Invert the petri plate and incubate for 24 hours.
5. Observe, record, and interpret results.

bacterial species	growth (+/-)	salt resistance and mannitol utilization (+/-)	medium color surrounding growth	mannitol fermentation (+/-)
Staphylococcus aureus				
Staphylococcus epidermidis				

Questions

1. Fill in the table above with results and interpretations.

2. Define selective medium.

3. Explain how mannitol salts agar is a selective medium.

4. Define differential medium.

5. Explain how mannitol salts agar is a differential medium.

6. Explain how phenol red, a component of mannitol salts agar medium, detects if a species conducts mannitol fermentation.

Chapter 14 Eosin-Methylene Blue (EMB) Medium

Objectives

- Identify that EMB medium is both selective and differential.
- Explain how EMB is a selective medium and how this works.
- Explain how EMB is differential and how this works.
- Define coliform.
- Successfully utilize EMB medium and interpret its results.

Introduction

Eosin-methylene blue (EMB) medium is a **selective medium** and a **differential medium** used for isolation of Gram-negative rods in a variety of specimen types, including being used frequently in clinical laboratories. EMB is a selective medium because it inhibits Gram-positive bacterial growth. The two dyes in this medium, eosin and methylene blue, act as selective agents to inhibit Gram-positive bacteria, but allow for the growth of Gram-negative bacteria.

EMB enables differentiation of bacterial species that are **coliforms** due to its dyes and its lactose content. EMB contains two types of sugars: lactose and sucrose. It is the lactose that is the key to this medium's ability to differentiate coliform bacteria. Lactose-fermenting bacteria (*Escherichia coli* and other coliforms) produce acid using lactose. When acids are produced by bacteria, the combination of the dyes (which serve as pH indicators in this medium) produces color variations in the colonies because of the acidity. Strong acidity produces a deep purple colony with a green metallic sheen, whereas less acidity may produce a brown-pink coloration of colony. Nonlactose fermenters appear as translucent or pink.

coliform: A classification of bacterial species that is typically associated with animal digestive tracts and fecal contamination in the environment. These bacterial species are Gram-negative bacilli that do not produce endospores and have the ß-galactosidase gene, and can therefore break down lactose. Coliform bacteria break down lactose to produce acid and gas.

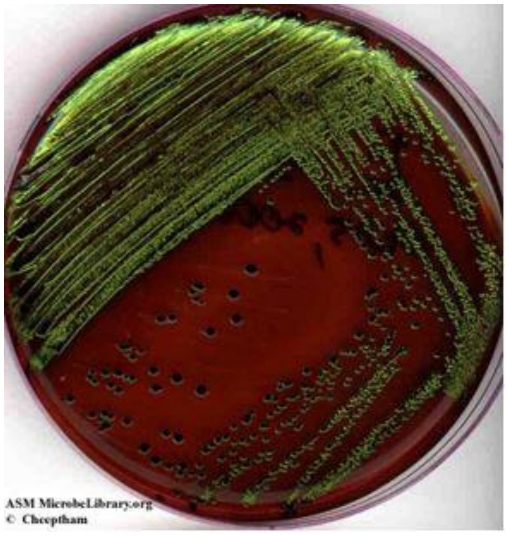


Figure 14.1: Eosin-methylene blue agar plate inoculated with *Escherichia coli* (a Gram-negative coliform bacterium) showing good growth of dark blue-black colonies with metallic green sheen indicating vigorous fermentation of lactose and acid production which precipitates the green metallic pigment.

Procedure

1. To observe the effects of EMB medium as selective and differential, aseptically conduct quadrant streak plates on three different EMB plates with:
   - *Escherichia coli* - a Gram-negative coliform species
   - *Pseudomonas aeruginosa* - a Gram-negative non-coliform species
   - *Staphylococcus aureus* - a Gram-positive species
2. Invert and incubate the plates for 24-48 hours.
3. Observe, record, and interpret the results.

bacterial species	growth (+/-)	species is likely Gram (+/-)	colony color(s)	lactose utilization produces acid (+/-)	species is a coliform (+/-)
Escherichia coli					
Pseudomonas aeruginosa					
Staphylococcus aureus					

1. Record the results and interpretations from the EMB plates in the table above.

2. What types of bacterial species is EMB used to identify?

3. How is EMB a selective medium? Take an image using your mobile.

4. How is EMB a differential medium?

5. Define coliform.

6. Explain the role of lactose in EMB.

Chapter 15 MacConkey agar (MAC)

Objective

- To understand Selective and differential medium
- To identify of Enterobacteriaceae

Introduction

MacConkey agar (MAC) is a bacterial culture medium named after bacteriologist Alfred T. MacConkey (1861-1931). MacConkey agar is a selective and differentiating agar that only grows gram-negative bacterial species; it can further differentiate the gram-negative organisms based on their lactose metabolism. The selective and differentiating properties of MacConkey agar enable utilization for both research and clinical applications. The fermentation of lactose produces organic acids, particularly lactic acid, which decreases the pH of the agar. MAC contains a pH indicator that turns pink under acidic conditions. Therefore, lactose-fermenting-gram-negatives (lactose-fermenters) will form pink colonies, while non-lactose fermenters will form off-white opaque colonies. Even within lactose-fermenters, species will show a varying rate of growth. The rate of growth is also a way to further differentiate organisms in the MAC medium. Lastly, some species that form a capsule appear differently.

Altogether, MacConkey agar only grows gram-negative bacteria, and those bacteria will appear differently based on their lactose fermenting ability as well as the rate of fermentation and the presence of a capsule or not. This makes MAC a powerful tool for differentiating and isolating bacterial species from the sample source. MAC is one of the many bacterial cultures clinical microbiologists utilize for diagnostic testing; this is still widely used in the clinical laboratory to identify causal agents from a patient (ie, stool sample).

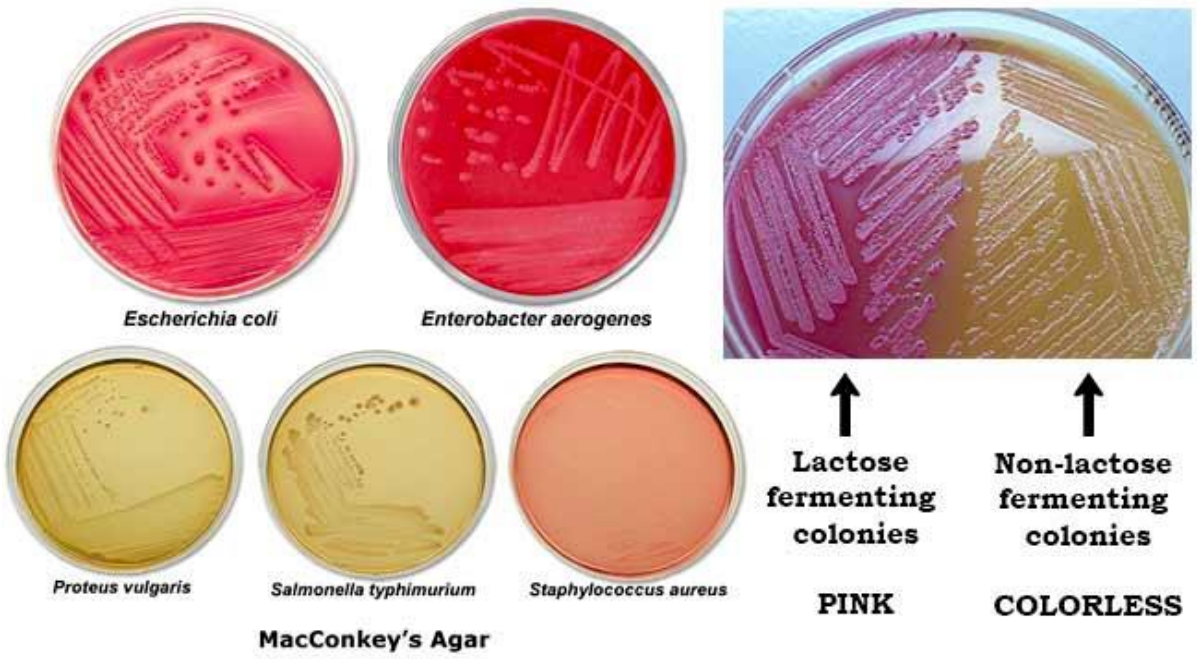


Figure 15.2 MacConkey Agar plates and Colony Morphology

Clinical Significance

A pure bacterial culture remains crucial to assess its virulence, its susceptibility to antibiotic therapy, and its genome sequence in order to facilitate the understanding and treatment of caused diseases. MAC's selective and differentiating properties as a culture medium allow isolating colonies of pure bacterial culture from a source sample. Typically in a clinical setting, the collected sample is placed on a panel of many growth media for identification and isolation of bacterial cultures. MAC is one of the widely used growth media as it can work to selectively grow gram-negative bacteria and further differentiate them based on their fermentation profile. Many pathogenic gram-negative bacteria can be differentiated by MAC, especially bacterial gastroenteritis-causing species. The diagnostic potential is immense. MAC is essentially a versatile foundation, in which additional substrates, such as sorbitol, can be incorporated for further differentiation. Another example is the addition of antibiotics for testing drug resistance. In intensive care units, testing multi-drug resistance in gram-negative bacteria is an important surveillance measure. Gram-negative enteric bacteria are a common cause of bacterial gastroenteritis, which is characterized by diarrhea, vomiting, and abdominal cramping. *Escherichia coli* and *Campylobacter jejuni* are some of the common causes of bacterial gastroenteritis. When bacterial gastroenteritis is suspected, the patient's specimen can be sampled and cultured on a panel of bacterial cultures, which includes MacConkey medium. MAC contributes to the identification of the causal agent by providing lactose-fermentation profiles in gram-negative species.

A pure bacterial culture remains essential for the study of its virulence, its antibiotic susceptibility,

and its genome sequence in order to facilitate the understanding and treatment of caused diseases. Bacterial cultures are still widely used laboratory techniques that many physicians rely on to make their final diagnoses. Many healthcare professionals are involved in this process, and proper communication and coordination will enhance patient-centered care, improve outcomes and patient safety, and enhance team performance. Bacterial culture from a patient's specimen is a commonly ordered test that many physicians use to aid their medical decision-making process. Nurses are frequently involved in obtaining and transporting samples from the patient to the laboratory. Clinical microbiologists must be trained with a thorough understanding of the principles to run the tests, interpret the results, and report the findings. Sterile sampling techniques and proper handling of the specimen during handling and transport of samples are required to avoid contamination. Any healthcare professionals handling these patient samples must be adequately trained in order to avoid contamination of the samples or accidental exposure and spread of potential pathogens.

Key components of the MacConkey medium include crystal violet dye, bile salts, lactose, and neutral red (pH indicator). Crystal violet dye and bile salts halt the growth of gram-positive bacteria. This allows only gram-negative species to form colonies on MAC agar. MacConkey agar contains the essential nutrients required for microorganism growth.

Additional key components include crystal violet dye, bile salts, lactose, and neutral red (a pH indicator). The lactose in the agar is a source of fermentation. Lactose-fermenting microorganisms will produce organic acids, particularly lactic acid, which will lower the pH. Neutral red is a pH indicator that turns from off-white to bright red/pink as the pH drops below 6.8.
Different species will yield colonies in varying appearances on a MacConkey medium based on their ability to ferment lactose. This gives McConkey agar its differentiating property.

- **Lactose (Lac) positive (pink colonies):**
  - Lactose-fermenting species will grow pink colonies. Lactose fermentation produces acidic byproducts that lower the pH, turning the pH indicator pink.
  - Examples of Lac-positive species: *Escherichia coli, Enterobacteria, Klebsiella*
- **Lac negative (white colonies)**
  - Gram-negative bacterial species will still form colonies, but colonies will have a white appearance as there will be no change in pH in the absence of lactose fermentation.
  - Examples of Lac-negative species: *Salmonella, Proteus, Yersinia, Pseudomonas*
- **No colonies:**
  - Gram-positive bacteria will not form any colonies on MacConkey medium.
- **Slow:**
  - Weak lactose fermenters will form colonies slower than the rest.

- Examples of slow lac fermenters: *Serratia, Citrobacter*

- **Mucoid: (sticky, wet colonies)**
-
  - Encapsulated bacteria produce capsules using lactose. This gives sticky, wet-appearing colonies.
  - Examples of mucoid colony-forming species: *Klebsiella, enterobacter*

1. Compare and contrast MAC and EMB.

2. Is it normal to use both MAC and EMB when identifying a bacterium? Why or why not?

3. What similarities and differences did you observe in your results with MAC and EMB?

4. What is the primary purpose of MSA?

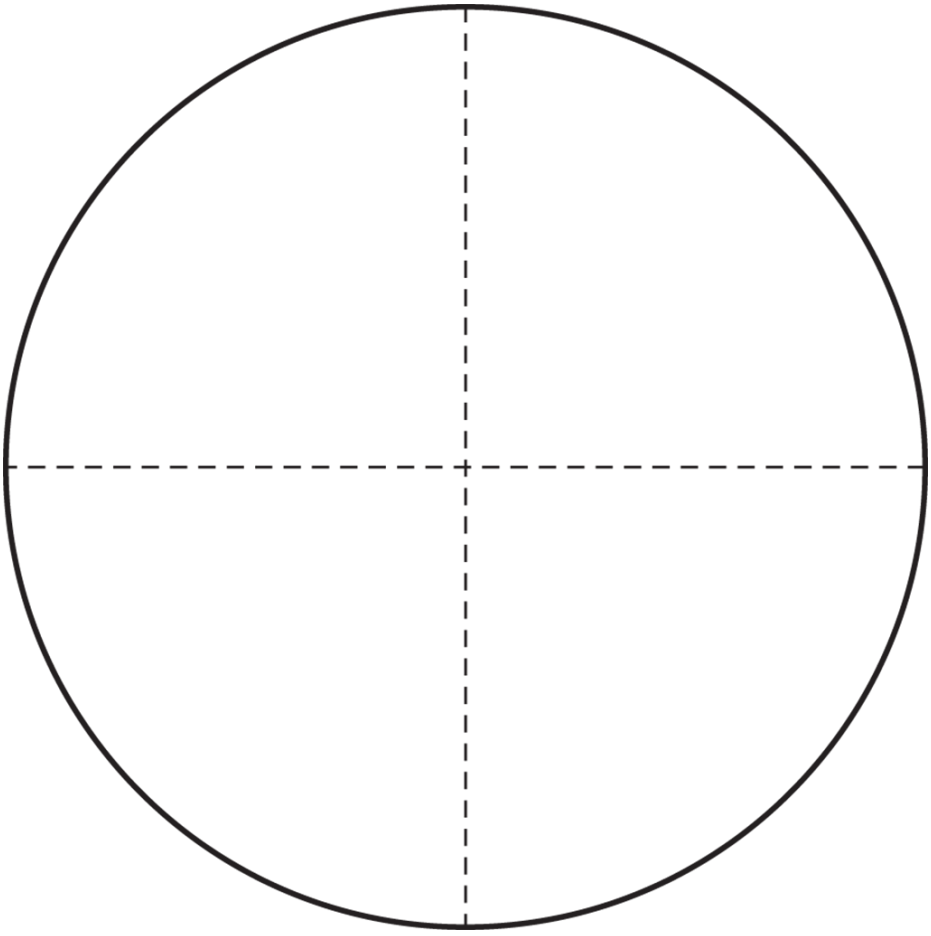
5. What differences between the different GPCs did you notice on MSA?

6. Why is MSA optional only during the unknowns?

As mention above that MacConkey agar (MAC) was the first solid differential media to be formulated which was developed at 20th century by Alfred Theodore MacConkey. MacConkey agar is a selective and differential media used for the isolation and differentiation of non-fastidious gram-negative rods, particularly members of the family Enterobacteriaceae and the genus *Pseudomonas*. Composition of MacConkey Agar is given on table below.

Ingredients	Amount
Peptone (Pancreatic digest of gelatin)	17 gm
Proteose peptone (meat and casein)	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm
Crystal Violet	0.001 g
Agar	13.5 gm
Distilled Water	Add to make 1 Liter

Draw your result on the circle and photograph with your mobile device. Take image for 4X, 10X, 40X and 100X and draw them on circle parts.



Chapter 16 Observation of Natural Samples

Objective: In this chapter, the major objective is going to be: To collect, observe, record, and identify microbes from natural samples.

Introduction

A course in microbiology most of the work done in an introductory microbiology course is in the laboratory, often with a slant toward the clinical world. However, microbes are found in all environments. Therefore, let's go outside. Let's find some microbes around college, grab some samples from natural sources outside the classroom and take a look. You will have opportunity to make live video and beautiful snaps or photos using your own iPHONE or smart phone.

Lets watch video together:

https://www.kickstarter.com/projects/1446839815/the-microbescope

1. Did you have difficulty using the your phone to observe specimen under microscope and/or did you find any organisms? Explain.

2. Which did you observe--Bacteria, Protists, Fungal, Animal microbes (rotifers, etc.)? Explain.

3. Draw and describe two microbes that you observed and give their major characteristics, size estimate.

Chapter 17 Catalase Test

Objective:

- To describe what catalase is and why it is important for bacterial survival.
- To give the chemical reaction catalyzed by catalase.
- To explain how testing for catalase is useful for characterizing and identifying bacterial species.
- To tell the types of bacterial species that typically produce catalase and those that typically do not produce catalase.
- To successfully conduct a catalase test and interpret results for a catalase test.

Introduction

Catalase is an enzyme produced by some species of bacteria. This enzyme protects bacteria from hydrogen peroxide (H_2O_2) that can damage and kill them. Catalase will convert hydrogen peroxide into liquid water (H_2O) and oxygen gas (O_2). As a result, if catalase is very active due to an abundance of hydrogen peroxide, the rapid production of oxygen gas (O_2) will produce bubbles.

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Figure 17. 1: The reaction catalyzed by the enzyme catalase. Two hydrogen peroxide molecules (2 H_2O_2) is converted into two water molecules (2 H_2O) and an oxygen molecule (O_2). "Catalase" is written over the reaction arrow to show that this is the enzyme that enables this chemical reaction to occur.

Bacteria that conduct aerobic metabolism (biological reactions that require O_2) produce hydrogen peroxide (H_2O_2) as a toxic byproduct of their metabolism. Toxic hydrogen peroxide can cause intracellular damage, such as damage to DNA, lipids, and proteins. To remove H_2O_2 and other similar compounds, cells produce enzymes to break them down, such as catalase.

Bacteria can only make catalase if they have the gene for catalase in their DNA. When the catalase gene is expressed by the cell (DNA-->RNA-->protein), the bacteria produce catalase. Only species that have the catalase gene will make catalase and will test **catalase positive**. Bacterial species that do not

have the catalase gene cannot make catalase and will test **catalase negative**. Therefore, testing for the presence or absence of catalase is a way bacterial species can be characterized and another tool for classifying and identifying bacterial species.

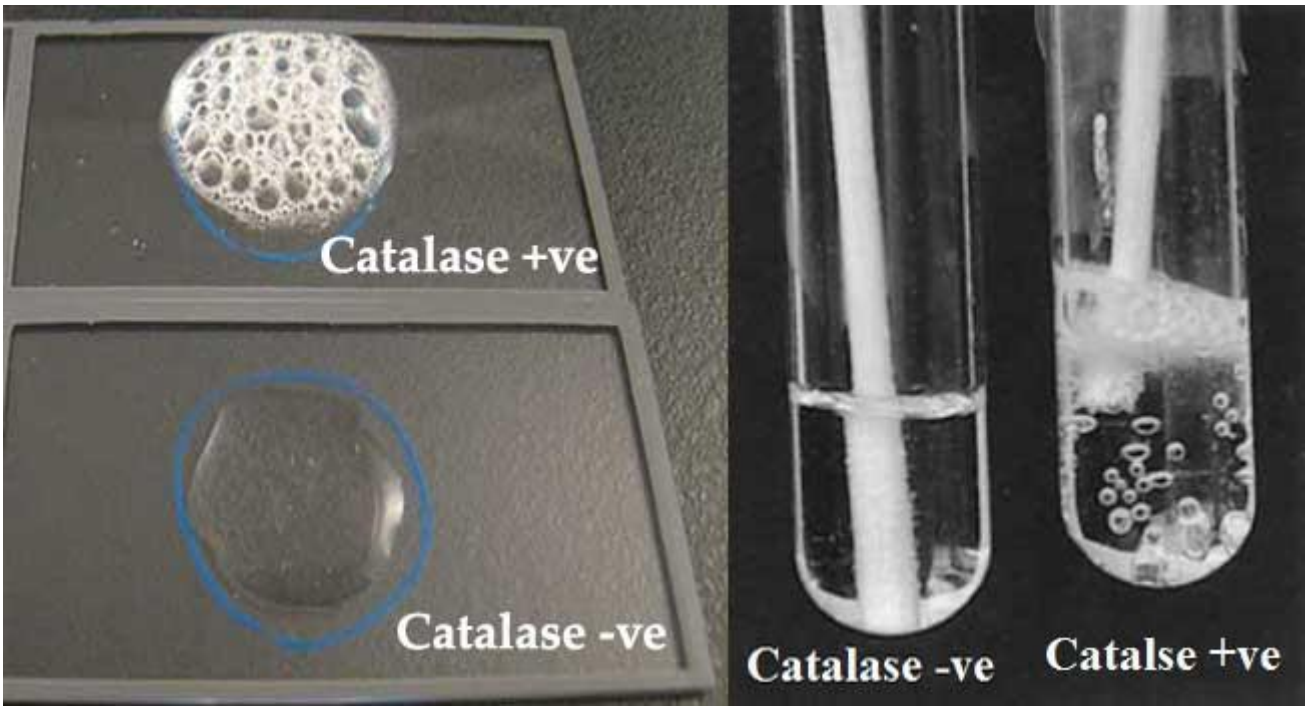


Figure 17. 2: Slide catalase test results. Hydrogen peroxide was added directly to bacteria placed on a microscope slide (left) and in test-tube (right). A catalase positive reaction is indicated by bubbling and negative reaction is indicated by lack of bubbling on slide and test-tube, respectively.
https://youtu.be/r3p4jHjw9o8

A simple test to determine if bacteria produce catalase is to add hydrogen peroxide to bacteria. This may be done by adding the hydrogen peroxide to bacteria placed on a slide or adding it to bacteria growing on an agar slant. If catalase is present, the hydrogen peroxide will be broken down into water and oxygen gas, resulting in the production of bubbles (catalase positive). This test does not require any special type of medium, however it should never be performed on organisms that have been grown on blood agar (a medium that contains blood). This is because there is a catalase activity in blood that would produce a false positive result.

Most aerobic bacteria (bacteria that require O_2) and facultatively anaerobic bacteria (bacteria that live and metabolize O_2 or in an environment without O_2) produce catalase. Obligate anaerobe bacterial species (bacteria that must live in an environment without O_2 to survive) lack catalase, which is why they cannot survive in an atmosphere containing oxygen. However, some of them have modified versions of catalase to deal with any possible exposure to oxygen.

Purpose or Uses of Catalase Test

- The morphologically similar *Enterococcus*or *Streptococcus* (catalase negative) and *Staphylococcus* (catalase positive) can be differentiated using the catalase test.
- Also valuable in differentiating aerobic and obligate anaerobic bacteria.
- Semiquantitative catalase test is used for the identification of Mycobacterium tuberculosis.
- It is used to differentiate aerotolerant strains of *Clostridium*, which are catalase negative, from *Bacillus* species, which are positive.
- Catalase test can be used as an aid to the identification of Enterobacteriaceae.

Questions

1. Complete the table above with results from the catalase test.

	Catalase (+/-)
Streptococcus pyogenes	
Staphylococcus aureus	

2. What is catalase?

3. Why is catalase production an advantage for bacteria to survive?

4. What types of bacteria typically produce catalase?

5. What types of bacteria typically do not produce catalase?

6. Give the chemical reaction catalyzed by the catalase enzyme.

7. When a bacterial species produces the catalase enzyme, what does it tell us about this species genes?

8. When a bacterial species does not produce the catalase enzyme, what does this tell us about this species genes?

9. Why can the catalase test be used to help with bacterial identification?

I Hope You Enjoyed!!!

Good Luck ahead for your career!!!!!!!