Laboratory 1. Laboratory introduction

OBJECTIVES: 1) Introduce basic laboratory techniques and procedures. 2) Learn basic microscope techniques used by plant pathologists. 3) Observe the occurrence of plant diseases in the natural environment and describe the common signs and symptoms.
SKILLS: 1) Basic safety procedures. 2) Two standard laboratory techniques for culturing microorganisms. 3) Basic microscopy procedures.

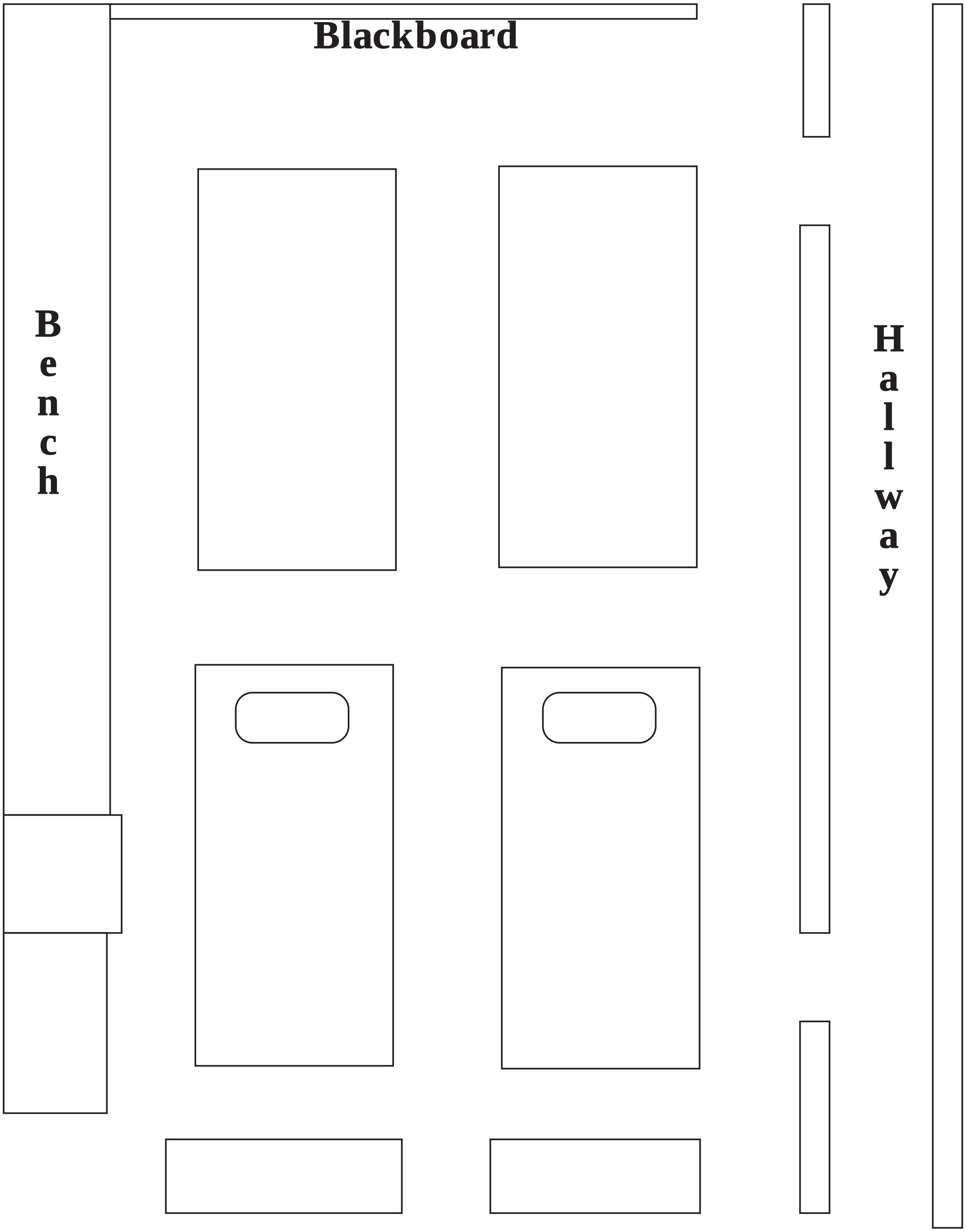
Exercise 1 – Lab Safety Scavenger Hunt (Group Activity)

PURPOSE: Safety protocols are necessary for safe operation of any laboratory. Familiarity with safety protocols reduces the likelihood of personal injury, limits contamination, ensures appropriate disposal of waste materials and allows effective management of emergencies. Because of this, we want you to be familiar with the location, function and operation of various pieces of safety equipment.

On the next page is an illustration of the lab room and the adjoining hallway (overhead view). Working in your groups, find the safety items on the following list and draw in their locations on the illustration.

First Aid Kit
Safety shower
Eye wash (2)
Fire extinguisher (2)
Fire alarm
Sharps container (4)
Glass disposal container (4)
Biohazard waste container
Telephone
Nearest exit in case of fire

Lab Safety Scavenger Hunt Illustration



Exercise 2- Recognizing plant diseases with a campus disease tour (Class Activity)

PURPOSE: Plant diseases are not just found in the lab. Plant diseases cause physical, economic and aesthetic damage. Today we will observe plant diseases in YOUR natural environment, around the campus.

Please follow you instructor around campus and take notes on the diseases you observe. The pages below can be used for notes.

Disease 1
Name of host: _________________
Name of disease: __________________
Type of causal organism: ___________________
Symptoms and signs of disease:

Notes:

Disease 2
Name of host: _________________
Name of disease: __________________
Type of causal organism: ___________________
Symptoms and signs of disease:

Notes:

Disease 3
Name of host: _________________
Name of disease: __________________
Type of causal organism: ___________________
Symptoms and signs of disease:

Notes:

Disease 4
Name of host: _________________
Name of disease: __________________
Type of causal organism: ___________________
Symptoms and signs of disease:

Notes:

Disease 5
Name of host: ________________
Name of disease: _________________
Type of causal organism: __________________
Symptoms and signs of disease:

Notes:

Disease 6
Name of host: ________________
Name of disease: _________________
Type of causal organism: __________________
Symptoms and signs of disease:

Notes:

Disease 7
Name of host: ________________
Name of disease: _________________
Type of causal organism: __________________
Symptoms and signs of disease:

Notes:

Disease 8
Name of host: ________________
Name of disease: _________________
Type of causal organism: __________________
Symptoms and signs of disease:

Notes:

Disease 9
Name of host: ________________
Name of disease: _________________
Type of causal organism: __________________
Symptoms and signs of disease:

Notes

Growing Microorganisms in Culture

In nature, microorganisms do not exist in isolation from each other. To study a particular microorganism, you must first separate it and produce a pure culture (containing no other microorganisms). There are several ways to isolate your organism of interest, some of which are better suited to certain groups of microbes. All these methods require the use of microbiological culture media and sterile techniques. Many different culture media exist, and some are better suited to particular microorganisms than others; no single culture medium is suitable for all microbes. In fact, many microbes have not yet been grown on culture media.

Microbiological culture media provide essential substances including inorganic salts, a carbon source for energy (often a sugar, such as sucrose or glucose) and various other organic molecules necessary for microbial growth, such as amino acids and vitamins. Many fungi and bacteria are capable of synthesizing all the cellular metabolites required for growth, and therefore can grow on media that simply provide a carbon and a nitrogen source.

Media can be made in either liquid (broth) or solid form. Broth can be solidified by adding agar (usually at 2%), a non-utilized carbohydrate from marine algae. Much like gelatin, agar added to a liquid medium is usually dissolved by heating to boiling. Media, once prepared, is sterilized (described below), cooled to about 60°C, and dispensed into sterilized Petri dishes (covered plates). Molten agar solidifies at about 40-45°C.

Types of culture media

Complex media - Complex media contain undefined compounds. Potato Dextrose Agar (PDA) and Nutrient Agar are examples of complex media used in this lab. We do not know the exact chemical constituents of either the potatoes or the nutrient broth. PDA is a general purpose media for fungi, but bacteria will also grow on it.

Selective media - Selective media are used to isolate a target organism, and no other organisms. The media are designed to enhance growth of the target organism while inhibiting growth of non-target organisms (contaminants). For example, lactic acid can be added to PDA to make acidified PDA (APDA). The lower pH of APDA (compared to PDA) promotes growth of fungi in preference to bacteria.

Differential media - A differential medium differentiates between the target organism and all others that may grow on that particular medium.

- The medium may contain a dye that only the target organism can take up. Thus, colonies of the target organism will be stained by the dye, while other organisms will not be stained. MacConkey's agar contains a dye which stains colonies of *Erwinia* spp. pink, while other bacterial colonies remain white.
- The medium may enhance particular characteristics of the target organism. King's B promotes the production of diffusible, water-soluble, UV-fluorescent pigments of some *Pseudomonas* spp.

Synthetic media (or defined media) - Synthetic media have a defined set of chemical constituents. The exact compounds and their exact amounts are known. We will not use any synthetic media.

Avoiding contamination
In order to avoid contamination of microbial cultures, it is necessary to use sterile media and tools. Whilst you are working, sterile media must be kept free from contaminating microbes carried in the atmosphere, or on instruments used in transferring microbes, by using "sterile technique". Methods for sterilization and sterile technique are described below.

Sterilization of media and tools
Autoclaving - An autoclave uses superheated steam to kill microorganisms. The autoclave reaches temperatures of 121°C at 15 psi pressure, and these conditions are maintained for 15-20 minutes. It is the elevated temperature and not the higher pressure that kills the microbes. This is sufficient to kill even the endospores formed by some bacteria, which can survive boiling. Culture media, glassware and tools (e.g., transfer loops, razor blades) are sterilized by autoclaving.

Working with "sterile technique"

Laminar Flow Hood - The laminar flow hood, in which air is forced through many layers of fine filters to exclude airborne contaminants, provides a sterile environment in which to pour culture media, prepare cultures, and transfer cultures to fresh media. Also, if one wishes to use media which have been stored in a refrigerator, the sterile hood provides a space to dry off the water which condenses on the plates as they warm to room temperature.

Working at the bench - Sometimes, culture work must be done in an open room, as in this laboratory. Under these circumstances additional precautions are necessary. Before you do any culture work in this laboratory, wipe your bench top working surface with a 10% bleach solution. If you wish, cover your working area with a layer paper towels moistened with 10% bleach. TAKE CAUTION THAT YOU DO NOT BLEACH YOUR CLOTHES OR GET BLEACH INTO YOUR EYES. Instead of bleach, you can wet the toweling with water.

Sterilization Methods for Tools

Flame Sterilization – Hold the end of the tool in the tip of the lower cone of the flame of a Bunsen burner until the tool is red-hot (your lab instructor will show you how to do this). Remove the tool and allow it to cool for about 10 seconds or by touching it to sterile agar several times. Cooling is important as you don't want to fry your culture!

Alcohol Sterilization – Dip the end of the tool in alcohol (95% EtOH) and then pass it briefly through a flame. You will see the alcohol burn off from the tool. Wait a few seconds to allow the tool to cool before you use it on your culture. Note that the alcohol, not the flame, sterilizes the tool, so it is not necessary to hold the tool in the flame.

Exercise 3 – Standard techniques used for culturing microorganisms (Individual Activity)

PURPOSE: Learn some basic methods used to study plant associated microorganisms.

Three-way streak - used to produce a pure culture of bacteria on solid media

Materials:

Petri plate with bacterial culture
Two sterile Petri plates with nutrient agar (NA)
10% Clorox bleach
Materials for sterile transfer: 95% ethanol, razor blades, paper towels, cheesecloth, sterile water, cutting board, forceps, matches, transfer loop

Protocol:

1) Before starting the three-way streak protocol, find out what microbes live on and around you! Label one of the sterile Petri plates with your name and today's date. Then, open the lid of the Petri plate, and either touch the medium, cough on it, or touch it with an object you find on the bench, such as a non-sterile paper towel. Record what you did in your notes and on the plate.

2) Prepare your work area for sterile work by wiping down the bench top with a 10% bleach solution.

3) Label the second sterile Petri plate with your name, today's date, and "3-way streak".

4) Flame the loop end of a transfer loop until red-hot. Wait until the loop cools. Do not wave the loop to cool it. Why not?

5) Open the top of the Petri plate that contains a bacterial culture. Pick up a colony with the sterile loop.

6) Open the top of the sterile Petri plate just enough to insert the transfer loop. Densely streak one-third of the plate. Lower the cover.

7) Rotate the plate one-third turn counterclockwise. Flame and cool the loop again.

8) Open the top of the Petri plate just enough to insert the transfer loop. Pull loop through dense inoculum area once and then streak another one-third of the plate. Lower the cover.

9) Rotate the plate another one-third turn counterclockwise. Flame and cool the loop again.

10) Pull loop through the second streak and then streak the rest of the plate.

11) Seal both plates with Parafilm. Incubate them upside-down in your lab drawer until next week.

What do you expect to see next week?

How does the three-way streak allow you to isolate a pure culture?

Construction of a moist chamber - used to induce sporulation of fungi

Materials:

Diseased leaf
Sterile, empty glass Petri plate, containing a marked piece of filter paper
Sterile water
10% Clorox bleach
Eppendorf tubes
Materials for sterile transfer: 95% ethanol, razor blades, paper towels, cheesecloth, sterile water, cutting board, forceps, matches

Protocol:

1) Prepare/sterilize work area as before. Label Eppendorf tubes “water”, “1 min” and “5 min”.

2) Divide plant sample into three portions, ensuring that each portion includes some diseased tissue. Place one sample into an Eppendorf tube with 1 ml of distilled water. The other two samples will be treated with 10% Clorox: one for 1 min, and the other for 5 min.

What effect do you think the Clorox will have? What differences do you think you will see between the three treatments?

3) After Clorox treatment, rinse plant samples twice in sterile water, by carefully tipping out the liquid (retaining the leaf sample) and replacing it with sterile water.

4) Label the glass Petri plate with your name and today’s date.

8) Add sterile water into the Petri plate so that the filter paper is completely moistened but not standing in water. (It is easier to add more water than to remove excess water, so be frugal!)

5) Flame-sterilize a razor blade. Soak the razor blade in ethanol for at least one minute. Remove it with forceps, and then use the flame to ignite and burn off the ethanol. Do not hold razor blade in the flame. Let the razor blade COOL.

6) Using the flame sterilized razor blade and a cutting board, cut a square of leaf tissue from one of the treated leaf samples (include some diseased tissue).

7) Flame-sterilize your forceps (same techniques as for razor blade) and allow them to cool. Transfer cut leaf tissue to the filter paper using flame sterilized forceps. Repeat for each plant sample, making sure that you flame sterilize your tools in between different samples.

8) Seal the plate with Parafilm and store in your lab drawer right side up till next week’s lab.

Use of Microscopes in Plant Pathology

Not all symptoms and signs can be observed macroscopically. Since most plant pathogens are microorganisms and their signs are microscopic, microscopes are an essential tool for plant disease diagnosis. The two types of microscopes most commonly used for general diagnosis are the dissecting microscope (sometimes called a stereoscope) and the compound microscope. Both will be used extensively throughout this class. The following exercise is intended for you to observe microscopic symptoms of plant diseases as well as for you to review the parts and the proper use of the dissecting and compound microscopes.

Theory of Microscope Operation

The compound microscope is an optical instrument in which a double magnification takes place. Light passing through the specimen on the stage is gathered by the objective producing an image that is magnified in proportion to the properties of the objective. This image is then picked up by the ocular lens (eyepiece), magnified a second time, and transmitted to the eye of the observer. The image seen by the observer when looking through the ocular lens has been magnified twice, once by the objective and once by the ocular. Total magnification of an image viewed through the microscope is calculated by multiplying the objective magnification and the ocular magnification. There is a number on each of the objectives (4X, 10X, 40X, 100X) and the oculars (10X, 15X) indicating the magnification for that particular lens set. For example, the image that you see with a compound microscope using the 40X objective with a 10X ocular would be magnified by a total of 400 times (400X).

While the magnification is important in microscopy, it is not the only factor to consider. It was previously thought that magnification by an objective lens could be increased without limitation by the addition of lenses. The nature of light and the properties of lenses prevent this. When magnification is increased to extremes, the resulting image is not necessarily clear, undistorted, or easy to interpret. Another set of factors must be taken into account regarding image clarity, or resolution (sometimes called resolving power). Resolving power is the ability of an optical system to enable the observer to distinguish as separate two points that are very close together. Resolution is determined by both the wavelength of light used for illumination and the physical properties of the lenses. Under optimal conditions (using green light and an oil immersion objective lens), the smallest resolvable object that can be seen with a good compound microscope is about 0.00022 mm (0.22 μm) in diameter. It is of no value to increase the magnification to great extremes when resolution of the image is lost. The maximum useful magnification for the ordinary light microscope is about 1,000 diameters (oil immersion objective lens).

Characteristics of the Optical System

The compound microscope that you will be using has many of the features of the finest research-quality microscopes. Below is a table of some of the characteristics of the objective lenses on your microscope.

Objective lens Magnification	4X	10X	40X	100X*
Working Distance (mm)**	19.87	5.40	0.39	0.11
Resolving Power *** (μm)	3.4	1.3	0.52	0.26

*Oil Immersion objective.
**The distance from the specimen or cover glass to the nearest point of the objective.
***The smallest distance between two points visually distinguishable when viewed through the lens.

You will be assigned a dissecting and a compound scope to be used throughout this class. Record the numbers of each microscope below.

Dissecting scope No. __________ **Compound scope No. __________**

General microscope care

1) Carry the microscope in an upright position. Hold the vertical stand in one hand and support the microscope base with the other.

2) Place the microscope at least 4 inches from the edge of the lab bench. Adjust your chair for comfortable viewing.

3) Lens elements can be very easily scratched, even during routine cleaning procedures. Clean microscope lenses by gently brushing them with a camelhair brush. If necessary, gently wipe the lens with a new piece of lens paper. If the debris remains, gently wipe the lens with a new piece of lens paper that has been moistened with water.

Do not use alcohol or other organic solvents to clean the lens!
Organic solvents can dissolve the glue holding lens elements together.
Do not use paper towels, tissues, or cloth to wipe lenses!
Anything other than lens tissue can easily scratch lenses.

4) Any parts of the microscope that are meant to be turned are knurled or grooved.

5) Before plugging in any microscope, make certain that the light switches are in the off position, and that the variable light intensity rheostats are at the position for lowest illumination.

Parts and operation of the dissecting microscope

On this photograph, correctly label the microscope parts indicated below in bold type.

1) The dissecting microscope has two light sources: a **side-mounted light source** for using reflected light and the other under **the microscope base** for transmitted light.

2) Place a specimen on **the stage**. Turn on the light and adjust it so that the specimen is illuminated.

3) Look through the **eyepieces** and focus on the specimen. Adjust the distance between the two eyepieces so that you can comfortably use the full field of view. Close your left eye and turn the **focus knob** to bring the specimen into exact focus for your right eye. Next close your right eye and turn the ring around the left eyepiece, not the focus knob, to bring the specimen into focus for your left eye.

4) Change magnification by turning the **magnification knob** on top of the body of the scope.

5) When you are finished using the dissecting microscope for the day:

1. Turn light sources off
2. Clean stage with a moist paper towel
3. Wrap up cord, place cover over the scope
4. Return to the proper location in the cabinet

Exercise 4- Observation of symptoms and signs with the dissecting microscope (Individual Activity)

PURPOSE: Learn to use microscopes to examine plant materials for disease symptoms.

Examine samples of plants with the dissecting microscope. Obtain a diseased plant sample from those marked for use with the dissecting microscope. First, observe the sample and describe any symptoms you see with your unaided eyes. Then, observe your sample using the dissecting scope. Again, describe the symptoms you see.

Plant name: ____________________

With the unaided eye:
Describe symptom and signs ____

Drawings

With the dissecting scope:
Describe symptoms and signs ____

Drawings

Parts and operation of the compound microscope:

On this photograph, correctly label the microscope parts indicated below in bold type.

1) Using the **coarse focus knob**, lower the **mechanical stage** as far as possible. Place temporary slide mount within the frame on the stage.

2) Make certain that the **light switch** (front of base) is off, and **variable illumination control** (right side of base) is all the way towards you. Turn on the light and adjust variable illumination control so that light is coming through the condenser. Adjust the **condenser** by raising it all the way up and then lowering it by 1/8 to 1/4 inch.

3) Using the knurled ring, turn the **revolving nosepiece** to the 4X objective. Do not touch the objective itself. Observing from the side, turn the coarse focus knob so that the **objective lens** is slightly above the slide surface. Be careful that the objective lens does not contact the slide.

4) Looking through the **eyepieces** carefully lower the mechanical stage using the coarse focus knob to bring the image into rough focus. Use the **fine focus knob** to bring the image into sharp focus.

5) To increase image magnification (after the specimen is in focus at a lower magnification) carefully rotate the revolving nosepiece to bring the desired higher power objective into position. Focus the image using only the fine focus knob. If it is necessary to bring the stage up towards a lens, do so only while observing from the side, not while looking through the lens.

6) Do not use the **oil immersion lens** in this lab. Oil immersion lens will be used when we examine bacteria. Because of its close working distance, special care must be taken when using the 100X oil-immersion objective. First focus the specimen with the 40X objective lens in place. Rotate the 40X objective to the side and place a drop of immersion oil on the slide. While watching from the side, carefully rotate the100X oil-immersion lens so it is touching the oil drop. Focus using only the fine focus knob. Be careful not to let the 100X objective contact the slide.

7) When you are finished using the compound microscope for the day:

1. Rotate nosepiece to 4X objective
2. Use lens paper to clean the oil immersion lens.
3. Turn light control level all the way down, turn light switch off
4. Center and lower the stage
5. Remove slide from stage
6. If slide is a permanent preparation, clean it with lens paper
7. Wrap-up cord, place cover over the scope
8. Return to the proper location in the cabinet

Exercise 5 – Wet mount preparation (Individual Activity)

PURPOSE: Learn to use microscopes to examine signs of plant disease.

Using spore samples provided, prepare a wet mount of each specimen using the following procedure.

1. Place a drop of water on a clean microscope slide.
2. With a dissecting needle or other tool, pick up a spore sample and place into the water drop.
3. Touch one end of cover slip to the edge of the water drop and hold at a 45° angle, supported by forceps or dissecting needle.
4. Slowly lower the cover slip over the droplet.
5. Remove excess water by wicking it away from the cover slip edge with a piece of tissue paper.

Spore sample 1:

Sample name ___________________________

Magnification ___________________________

Draw structure

Spore sample 2:

Sample name ___________________________

Magnification ___________________________

Draw structure

Lab 1 Study Questions

TERMINOLOGY: Define the following terms

sterile technique –
culture media –
autoclave –
objective lens –
sporulation –
bacterium –
fungus –
pathogen –
resolution –
magnification -
wet-mount

QUESTIONS:

1) What is the purpose of performing the three-way streak

2) Why would placing a leaf that has a fungal disease in a moist chamber cause sporulation?

3) List one use of the following safety items.

safety shower

eye wash

sharps container

biohazard waste container

4) What are the advantages to using a dissecting microscope in disease diagnosis?

5) What are the advantages to using a compound microscope?

6) What is the total magnification of an object viewed with 40X objective and 15X oculars?