Lab 3 - Introduction to Microscopy

A. Objectives

1. Identify the parts of the compound microscope;
2. Discuss the function of each part of the compound microscope;
3. Learn to use compound microscopes without damaging any parts;
4. Learn how compound microscopes work;
5. Understand the concept of maximizing resolution.

B. Before coming to lab

Read this laboratory exercise and pp. 94-97 in Campbell et al. *Biology.* You do not need to prepare a protocol. However, there will be a quiz on microscope care at the beginning of lab.

C. During lab

During your instructor's demonstration, fill in as many blanks in the worksheets in Parts F and G as possible.
Carry out the exercise as described under Part H.

D. After lab

1. Fill in the remaining blanks in the worksheet in Part F. You may consult the following web site to see some interactive tutorials on microscopy: http://www.olympusmicro.com/primer/java/index.html (accessed 7/06). Review the information and worksheet in Part F to prepare for the quiz on microscope care.

E. Background

(adapted with permission from Heidcamp, W. H. Online cell biology laboratory manual**)**

Since its invention, the microscope has been a valuable tool in the development of scientific theory. Magnifying lenses have been known for as long as recorded history, but it was not until the advent of the modern compound light microscope that the device was used in biology.

A compound microscope is composed of two elements; a primary magnifying lens and a secondary lens system, similar to a telescope. Light is caused to pass through an object and is then focused by the primary and secondary lens. If the beam of light is replaced by an electron beam, the microscope becomes a transmission electron microscope. If light is bounced off of the object instead of passing through, the light microscope becomes a dissecting scope. If electrons are bounced off of the object in a scanned pattern, the instrument becomes a scanning electron microscope.

The function of any microscope is to enhance **resolution**. The microscope is used to create an enlarged view of an object such that we can observe details not otherwise possible with the human eye. Because of the enlargement, resolution is often confused with **magnification**, which refers to the size of an image. In general, the greater the magnification, the greater the resolution, but this is not always true. There are several practical limitations of lens design which can result in increased magnification without increased resolution. Figure 1 illustrates this point.

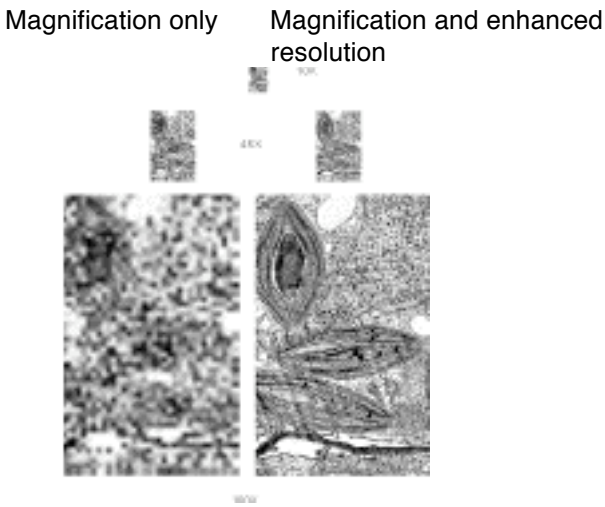

Figure 1: Magnification versus resolution

If an image of a cell is magnified from 10x to 45x, the image gets larger, but not necessarily any clearer. The image on the left is magnified with no increase in resolution. The image on the right is magnified the same, but with increasing resolution. Note that by the time the image is magnified 10x (from 10x to 100x), the image on the left is completely unusable. The image on the right, however, presents more detailed information. Without resolution, no matter how much the image is magnified, the amount of observable detail is fixed, and regardless of how much you increase the size of the image, no more detail can be seen. At this point, you will have reached the limit of resolution or the resolving power of the lens. This property of the lens is fixed by the design and construction of the lens. To change the resolution, a different lens is often the only answer.

The reason for a dichotomy between magnification and resolution is the ability of the human eye to see two objects. It is necessary that two objects be about 0.1 mm apart when held 10" from the face in order for us to detect them as two objects. If they are closer than 0.1 mm, we will perceive them as a single object. If two objects are 0.01 mm apart, we can not detect them unless we magnify an image of them by 10x. What has happened is that we have effectively altered our resolution ability from 0.1 mm to 0.01 mm through the use of a magnifying lens. We would say that our limit of resolution has changed from 0.1 mm to 0.01 mm, or inversely, our resolving power (resolution) has increased by a factor of 10.

Unfortunately, a lens can magnify an image without increasing the resolution. Several artifacts can be inherent in the lens design which cause the objects to become blurry at the edges. Thus, even though they can be made to appear 0.1 mm apart, the edges are so blurry that we lose the ability to see them as two objects. Think of a standard eye chart: you can see the increased size of a letter, but may be unable to tell what letter is projected.

Figure 1 illustrates what can be seen with increased magnification and resolution. If we were to look only at the left side of the figure, we could get the impression that the cell is filled with a homogeneous fluid (cytoplasm). If, however, we look at the right side of the figure, it becomes apparent that the cytoplasm is actually composed of smaller particulate components (chloroplasts, ribosomes, membranes). As we increased the resolution of our microscopes we

changed our concepts from protoplasm (the fluid of life) to cytoplasm (the fluid of the cell outside of the nucleus) to a highly ordered machine full of individual organelles.

It is readily apparent that while microscope lenses are usually discussed in terms of their magnification, the most important value is their resolution. All microscopes will come with a lens that can magnify 40 times the normal size, but only a quality lens will allow you to see more than you would with a good hand-held magnifying lens.

You can determine the resolving power (and thus, the quality) of your lens with the following equation. *Note that a **small** value indicates high resolution, because you want to distinguish two points that are a **small** distance apart*

$$d = (0.61\ \lambda)/N.A.$$

d = resolution, resolving power
0.61 = a constant
λ = wavelength of light (color)
N.A.. = numerical aperture (a measure of the light-gathering capacity of a lens)

The numerical aperture of a lens is defined as

$$N.A. = n \sin \alpha$$

n = refractive index of the air or fluid medium between the specimen and the objective lens
α = half-angle of the cone of light (angular aperture) entering the objective lens from the specimen (see Fig. 2)

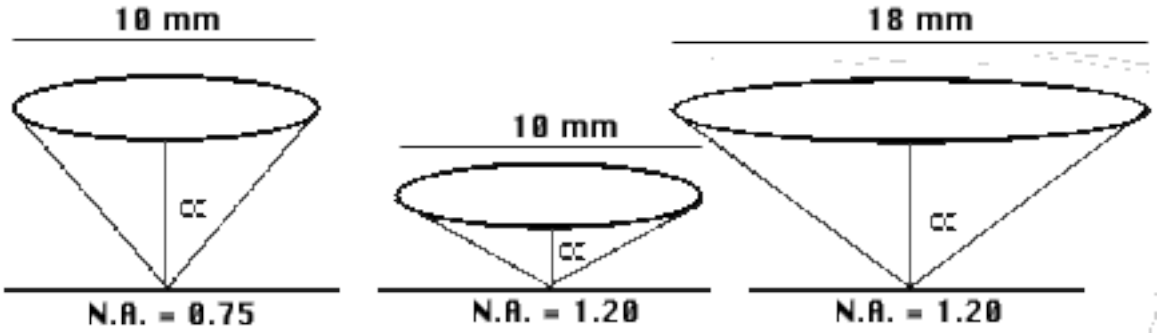

Figure 2.: Numerical apertures for various angles of incidence

The refractive properties of a lens are summed up in a measurement known as the refractive index (R.I. or n). The refractive index is a function of the bending of light from air through glass and back again. In a microscope, the glass of the lens is specially formulated to increase its refractive index. Once manufactured this property cannot be changed. The media around the lens can be altered, however, by removing air from between the objective and the slide, and replacing it with immersion oil.

Putting all of this to practical use, it is apparent that resolution can be increased in three ways. The easiest method is to increase the angle of light incidence, by altering the position and/or design of the substage condenser. Second, the refractive index can be maximized by using

specially manufactured lenses, and by controlling the medium through which the light travels, i.e. using immersion oil with lenses designed for this purpose. The third method is to decrease the wavelength of light used. For practical purposes, the wavelength has a larger effect on resolution than either changes in the angle of incidence or the refractive index. For maximum resolution, all three properties must be optimized.

For routine bright field microscopy, it is more convenient to work in the visible light range, and the shortest wavelength of visible light is blue. Thus, even inexpensive microscopes have incorporated a blue filter into their design, which is often referred to as a daylight filter. Resolution can be enhanced by reducing the wavelength to the ultraviolet range and yet again by levels of magnitude to the wavelengths electrons have in motion. The use of electrons as the light source gives rise to the electron microscope. UV light can not be seen directly by the human eye (it will injure the retina of the eye) nor can we see electron beams. Thus, these forms of microscopy rely on photography, or upon fluorescent screens.

Even with sufficient magnification and resolution, a specimen can only be seen on a microscope slide if there is adequate **contrast** between the parts of the specimen. By closing the substage iris diaphragm below maximum usable aperture, you can improve contrast at the expense of resolution. Contrast is also often enhanced by using stains that bind differentially to different parts of the specimen.

Definitions

Total magnification	the product of the magnification of all lenses. In our case, the objective (usually 4x, 10x, 40x or 100x), ocular (usually 10x or 12x) and head magnification (1.25x) lenses.
Resolving power (d)	The least distance between points in the object that can theoretically be distinguished in the image. Resolving power is expressed in the same units as the wavelength of the light
Resolution	The distance between two points that can practically be distinguished under a given set of circumstances
λ	is the wavelength of light, visible light ~ 550 nm, blue is shorter (~ 450 nm), red light is longer
Depth of field	The thickness of the specimen, measured along the optical axis, which is in sharp focus for any one set of conditions
Numerical aperture (N.A.)	A function of the quality of the lens; a measure of the light-gathering capacity of the lens.
Refractive index (n)	Of air = 1; of oil = 1.5
α (half of total angular aperture)	Half the angle of the cone of light accepted by the lens when this angle is measured at the specimen. The substage condenser shapes the cone of light entering the objective lens. It can be regulated with the iris diaphragm, the working distance, and the distance between condenser and stage. Higher magnification objectives decrease the working distance, and increase the angular aperture (Fig. 2).

F. Worksheet: Microscope parts and functions

During your instructor's demonstration, fill in the missing information.

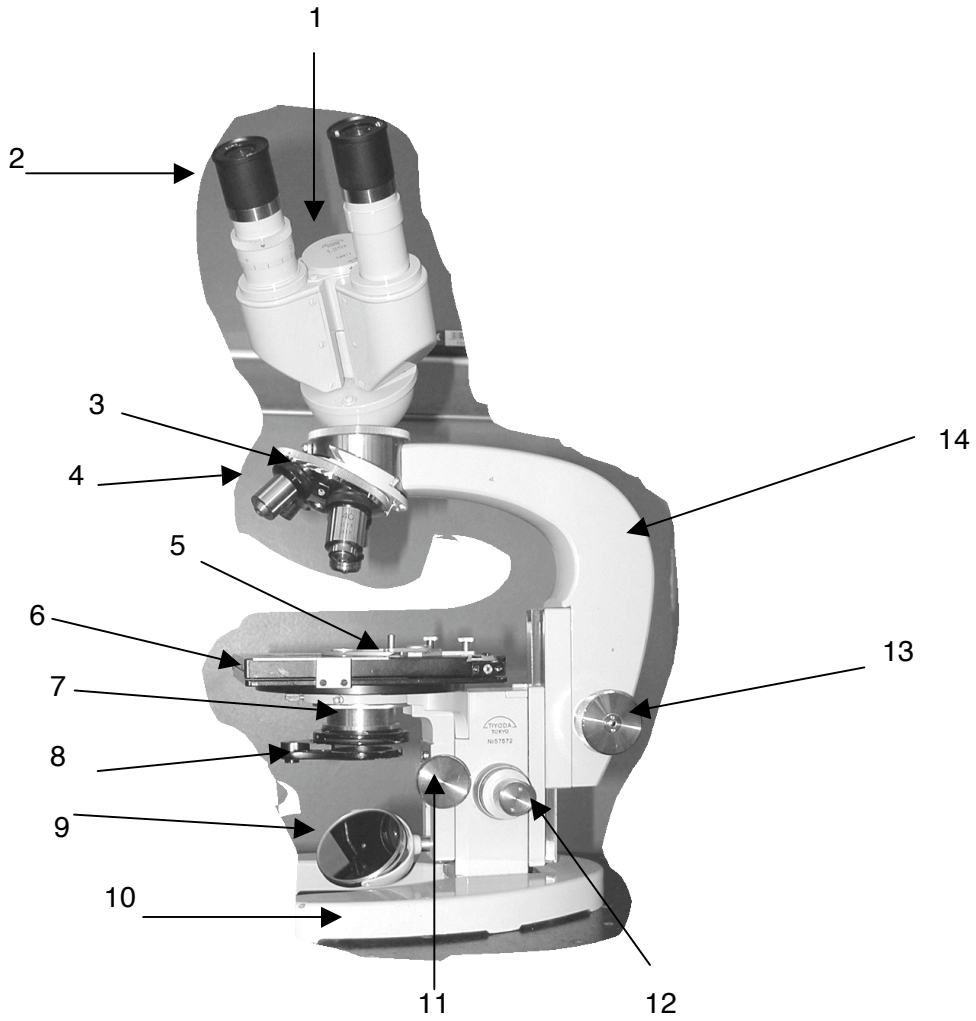

Figure 3: The parts of a compound microscope

1 _____

2 _____

3 _____

4 _____

5 _____

6 _____

7 _____

8 _____

9 _____

10 _____

11 _____

12 _____

13 _____

14 _____

Part	Function
Ocular lens	Projection of the lens system. All eyepieces will have a relative magnification written on the side of the barrel. The ocular lenses of our microscopes have a magnification of _____
Stage, stage clips	
Fine and course focus	
Condenser	
Objective lens 	Each objective has information critical for the maximum resolution possible written on the side of the barrel. Generally, the magnification is printed in the largest text. The second value is the numerical aperture. Beneath that, in a smaller font, the tube length and the cover glass thickness is given. The tube length, usually 160, refers to the distance between the objective lens and the eyepiece in millimeters. You can recognize a superior microscope if when adjusting the interpupillary distance you can see the eyepiece extend. This happens to maintain the proper tube length. The coverslip thickness, usually around .17mm, is also critical.
Nosepiece	
Substage iris (or aperture) diaphragm	**The iris diaphragm is the resolution versus contrast control.** It does this by varying the size of the numerical aperture of the objective lens. In addition, the iris diaphragm controls the depth of field.
Light source	
Interpupillary distance adjustment	

G. Microscope care

(adapted with permission from R. Griffin, City College of San Francisco)

Setting up your microscope and illuminator

1. Clear and clean your work area before getting a microscope.
2. Always carry the microscope in an upright position, because the ocular lenses are held in place only by gravity. Place one hand under the base and the other on the arm of the microscope. Be gentle when placing on your lab bench.
3. Always make sure that the cords of both the microscopes and illuminators are rolled up and secured so that you will not trip over them.
4. Never adjust screws, knobs or sliders without knowing their function. Never force any adjustment. If something does not move easily, ask your instructor for help.

Avoid lens contamination

Lenses are very expensive, they scratch easily. Dust, oil, and scratches impact your view of the object.

1. Avoid lens contamination by working with clean fingers and by putting a dust cover over the microscope before putting it back into its cabinet.
2. Clean the lense by blowing briskly across its surface. Then gently wipe the lense with dust-free lens paper. If the lens has oil on it, put a drop of the special oil remover on a piece of lens paper and wipe gently. Immediately wipe dry with dry lens paper. If you do not wipe it off immediately, oil removers like e.g., acetone can remove the cement that holds the lens in place.

Using your microscope

1. Always look from the side when changing objective lenses. When rotating the nosepiece, you can easily break a slide and scratch the objective lens.
2. Iris diaphragms consist of sliding leaves. They are easily damaged and expensive to repair. Always open or close the diaphragms slowly and gingerly. Never stick your fingers into a diaphragm.

Putting your microscope away

1. Turn illuminator off first to allow it to cool down. Put it away after everything else is put away.
2. Remove all specimen.
3. Check that lenses and all other microscope parts are clean (if they are not, clean them).
4. Adjust mechanical stage to minimum width.
5. Rotate nosepiece to bring lowest power objective (4x) into position.
6. Turn coarse adjustment knob to lower stage as far as it will go.
7. Place the dust cover on the microscope (if it has one), but reach under it to grasp the arm directly when you carry it.

Microscope care

Fill in the missing information.

Microscope care step	Reason
Clean workspace	
Hold microscope upright	
Hold with both hands, secure cords	
Watch from side if changing objective lenses	
Adjust mechanical stage to minimum width and lower arm before putting microscope away	
Never touch iris diaphragm with fingers.	

H. Exercise

1. Get a compound microscope and an illuminator from the cabinets by applying the safety rules outlined in the "microscope care" section. Place the illuminator so that light shines upwards into the condenser.
2. Sign the list for the microscope you checked out.
3. Obtain a prepared microscope slide with the letter e. Place the slide on the stage and ensure that it is locked in place with the slide holder (Figure 4 below)

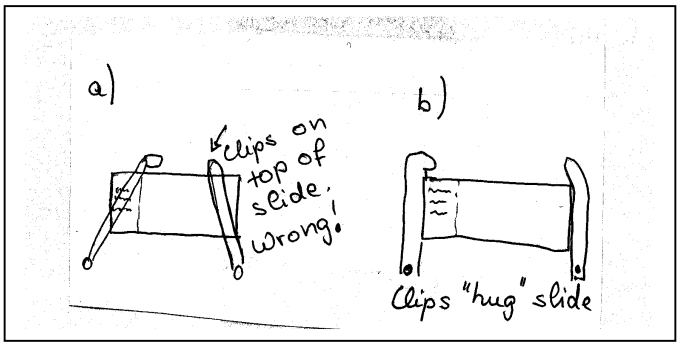

Fig. 4: a) wrong b) correct position of slide and stage clips

4. Rotate the condenser focusing knob to move the condenser to its highest position of travel. Although there is an ideal location for the condenser, the correct position of the condenser will vary slightly for each objective. Unless directed otherwise, it will not be necessary to move the condenser during any of the intended uses in this course.
5. Turn the illuminator on and adjust the light intensity to a comfortable level. Be sure that the condenser aperture is open if you have not set it as directed in the previous paragraph (slide the condenser diaphragm lever back and forth to check).
6. Looking down into the microscope, adjust the eyepieces to your interpupillary distance by pushing the eye tubes together or apart. Move the eye tubes back or forth until you see one uniform field of view.
7. The first time you use the microscope, adjust the eyepieces for your personal comfort. It is better to take your glasses off while viewing specimen with the microscopes we are using.
8. Get the blue filter out of the viewing path (if there is one). Click the 4x objective lens firmly into place.
9. Begin by focusing the microscope on any object within the field of view.
10. Find a suitably contrasted location in the center of the field of view and close your left eye. Using the coarse and fine adjustments, focus until you obtain a sharp image with your right eye only!
11. Now close your right eye and adjust the focus of the left eyepiece by rotating the diopter adjusting ring located on the left eyepiece. Do not readjust the focus of the left eye with the coarse or fine adjustments of the microscope - use the adjustment ring on the eye tube.
12. All subsequent uses of the same microscope will involve use of the coarse and fine focus adjustments, without reference to the procedures in steps 7 through 11.
13. Always begin focusing the microscope with the 4X magnification. Even if you are going to use the 100X, it is more efficient to begin with the 4X and then move up to the power desired. Objective lenses 10X, 40X, and 100X are parfocal, which means that if one is focused, each of the others is approximately in focus when revolved into position.

14. With the slide from Step 3 in place, rotate the coarse focus control until the slide is as close to the 4X objective as possible. Move the stage manipulators until a portion of the slide is directly under the objective and focus carefully on the object in view. After adjusting the focus at 4X, center the object to be viewed, and rotate the nosepiece to the next highest magnification. Since the 4X objective is not parfocal, you might need to use the coarse focus to focus your image when you switch to 10X. Use the fine focus control only once the 40X objective is in place.
15. Manipulate the fine focus to obtain the sharpest image. During use of the microscope, one hand should remain on the fine focus as constant readjustment will be called for. Use the other hand to manipulate stage movements.
16. Note that the microscope is typically designed so that one revolution of the fine focus knob raises or lowers the microscope stage 0.2 mm. This permits direct readings on the fine focus knob scale to 0.002 mm (2 microns) and can be used to determine the thickness of materials being examined.
17. Return to the 10X objective and move the slide around until you locate the letter e in the view. **In your notebook:** Note the orientation of the letter e on your slide and in the field of view.
18. To use the 40X objective, center the object you wish to view (the 40X will have a smaller field of view) and rotate the objective turret (referred to as the nosepiece) to bring the 40X objective into position. **In your notebook**: Is there any change in the orientation of the letter e?
19. Do not rotate the turret in such a manner as to bring the 100X into position.
20. **In your notebook:** Draw the image of the letter e at 10X (see “Drawing from microscope slides” below).
21. Write (draw) a protocol for making wet mounts of Elodea leaves according to your instructor’s demonstration.
22. Watch the demonstration of your laboratory instructor on how to prepare specimen for viewing with the compound microscope. Prepare wet mounts of Elodea leaves and onion epidermis. Use 1% acidified methyl green instead of water for the onion epidermis wet mount. Acidified methyl green will stain the nuclei. For each cell type, make a sketch at 40X and label as many of the cell structures in each drawing as you can.
23. Obtain a slide with crossed silk threads. Determine which color is on top, in the middle and on the bottom. Tool 4: Determining the relative vertical position of silk fibers. The depth of field is the thickness of the specimen that you can see in focus at one time. Because the depth of field is very short in the compound microscope, most often you have to focus up and down to clearly view all planes of a specimen. Find a good intersection where all three colors of fibers cross, but are well enough spread out that you can see the lower ones in the spaces between the upper ones. Choosing the correct objective is another secret to success, and you will find that the objective that gives the nicest looking picture may not be the best one for accomplishing this task. A third secret is to close the substage iris diaphragm a little more to make the yellow fibers clearly visible.
24. Report the result in your notebook and **let your instructor check your result**.
25. Put your microscope and illuminator away using the safety rules outlined in the previous lab section.

Drawing from microscope slides

1. All drawings must be completed in pencil. A #3 pencil is preferred since it will not smudge as readily as the standard #2. Colored pencils are optional. Ink is unacceptable.

2. Each drawing must be a **minimum of 10 x 12 cm** in size. Smaller drawings will not demonstrate sufficient detail, while much larger drawings require excessive time to fill in detail.
3. Drawings should be completed during the lab period in your lab notebook.
4. All labels should be added and appropriate indications of size should be made. Size is best indicated by including a ruler bar at the bottom of the drawing, drawn to scale and with the dimensions added (we will learn how to measure sizes later).
5. The size of your object should be proportional to the size of the field of view.
6. For the Elodea drawing: Sketch the outline of the cells proportional to the size of the field of view. Choose 2-3 representative cells to draw in detail.
7. On each drawing, place a line (bar) to indicate a length of 10 microns (next time).

F. Worksheet

1. What factors determine resolution?

2. Define “field of view”

3. Describe the principal precautions necessary when rotating the microscope nosepiece to change objective lenses.

4. Describe how to provide yourself with comfortable binocular vision through the microscope.

5. What is the maximum theoretical numerical aperture? (The maximum angular aperture for the best objective lenses is 70° (sin 70° = 0.94)

6. Use the value obtained above and calculate the limit of resolution (the smallest distance between two points that you can distinguish using the best objective lens available).

7. What is the highest resolution you can get using white light and no oil?