

**BIOLOGY 163 LABORATORY**  
**USE OF THE COMPOUND LIGHT MICROSCOPE**  
(Updated Fall 2005)

**Objectives**

1. To review/learn some of the basic principles of light microscopy.
2. To review/learn proper use of the compound light microscope, including proper focusing technique and set up of Kohler illumination.

Microscopes in various forms are important tools for biologists. Two types of microscopes that you will use frequently in this laboratory are the compound and dissecting light microscopes. Of these two, the compound microscope is more difficult to use well. Successful use of the compound light microscope depends on a variety of factors including quality of slide preparation, proper focusing, and adjustment for optimal illumination. The phase-contrast microscope is a special type of compound light microscope that you will also use in this laboratory. Additional information related to the phase-contrast microscope is provided in Appendix A.

**Materials**

You will work individually on this exercise. Each student will require the following equipment and supplies. Ask your instructor if you are not sure what something is or where to find it.

- Compound light microscope w/ Kohler illumination
- Prepared slides of letter "e" or other specimens

**SOME PRINCIPLES OF LIGHT MICROSCOPY**

A light microscope allows the user to enlarge the image (magnification) of the object being studied and to observe details of structure not detectable with one's unaided eye (resolving power) especially if the light is adjusted properly (Kohler illumination).

1. **Magnification.** Magnification with a light microscope is the product of the magnifying power of the objective lens times the magnifying power of the ocular lens. Compound light microscopes often have 4 objective lenses (4X, 10X, 40X, and 100X). The 4X, 10X and 40X objectives are called 'dry lenses' because they are designed to work in air. The 100X objective is an oil-immersion lens because its front element must be submerged in a drop of oil over the specimen. If the ocular lenses are 10X, the magnification of a specimen using the 10X objective would be 10X times 10X, or 100X.
2. **Resolving Power.** The resolving power of a light microscope is a measure of its ability to distinguish detail in a specimen. Resolving power is expressed as the **limit of resolution (lr)**. This is the smallest distance by which two neighboring points can be separated and still be seen as separate entities. The limit of resolution of your eye is between 0.1 and 0.2 mm. That of a good quality light microscope is about 0.2  $\mu\text{m}$  (1  $\mu\text{m}$  = 0.001 mm), a 500-1000 fold improvement over your eye. As the limit of resolution decreases, resolving power increases. The limit of resolution (lr) is calculated using Abbe's equation:

$$lr = \frac{0.61 \lambda}{n \sin \alpha}$$

where:  $\lambda$  = the wavelength of illumination used (see Fig. 1)  
 $n$  = the refractive index of the medium used between the objective lens and the specimen.  $n$  (air) = 1;  $n$  (oil) = 1.56  
 $\alpha$  = half the angle of light entering the objective lens (see Fig. 2); the theoretical maximum value of  $\sin \alpha$  is 1.

Notice that the limit of resolution becomes smaller (resolving power increases) as the numerator becomes smaller and/or the denominator becomes larger. With most compound light microscopes, you can manipulate the size of angle ( $\alpha$ ) and the value of  $n$ . The wavelength of light is fixed unless you add a colored filter to the light source.

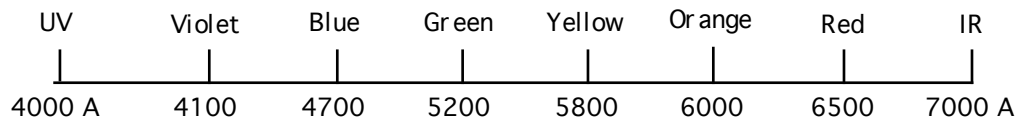


Figure 1. The Visible Light Spectrum

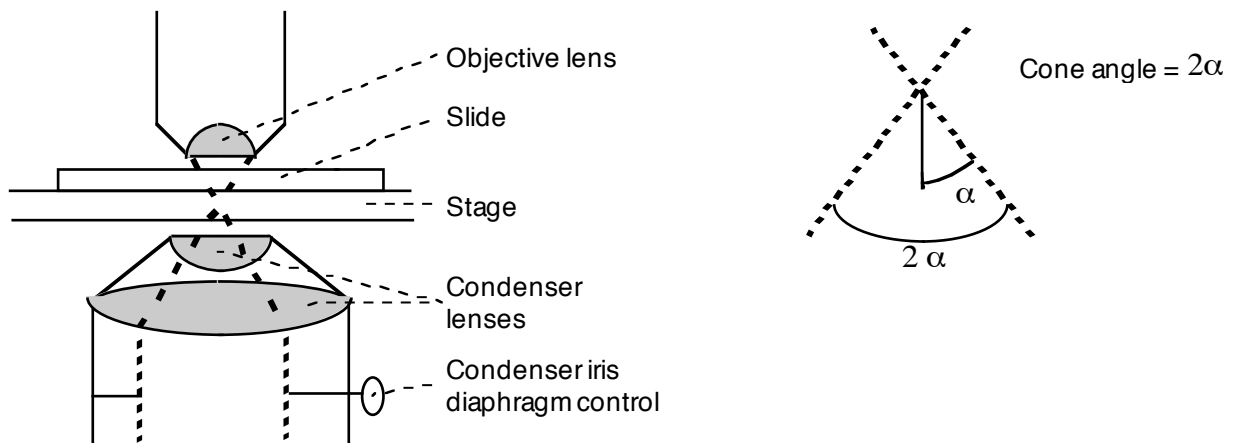


Figure 2. The angle of light entering the objective lens.

3. **Kohler Illumination.** In 1893, August Kohler described a method of setting up a compound light microscope to achieve the most uniform illumination from a non-uniform light source such as a light bulb. The result has become known as *Kohler illumination* and is obtained (1) by focusing the bulb filaments at the plane of the condenser diaphragm and (2) by focusing an image of the lamp diaphragm at the plane of the object. In microscopes with built-in illuminators the microscope manufacturer has done Step (1). You will need to do step (2) after some initial set-up procedures with the microscope.

## USING A LIGHT MICROSCOPE WITH KOHLER ILLUMINATION

### Some General Precautions

1. Check the instrument before starting your work to make sure all parts are intact and in good working order.
2. Carry the microscope by its arm with your other hand supporting the base.
3. **Do not tilt the microscope when carrying it. The ocular lenses may fall out.**
4. Do not remove objective lenses or take them apart. Clean the surfaces of lenses **ONLY** with lens paper.
5. **Good illumination is imperative.** Learn to obtain it soon. See "Using a Light Microscope with Kohler Illumination"
6. Always focus **upward** when the eye is at the eyepiece and you are using the coarse focus knob.
7. When rotating objectives on the compound microscope, be sure they do not contact the stage or slide.
8. Unless specifically instructed to do so, do not observe any material with the compound microscope unless the material has been placed in a proper fluid medium on a microscope slide and covered with a cover slip.
9. When using a compound microscope, always keep both eyes open.

When using a compound microscope you will have occasion to study both "wet" and "dry" preparations. The latter are prepared for you and consist of thin sections (about 0.01 mm thick) permanently set in some mounting medium. You make "wet" preparations to examine fresh material. Information on preparing wet-mount slides is provided for your reference in Appendix B. You will use "dry" preparations in this exercise.

### Procedure

1. Review the various parts of the microscope. You should be able to identify the ocular lenses, the objective lenses, arm, coarse and fine adjustment knobs, mechanical stage, the focusable condenser with diaphragm, and the built-in light source with diaphragm. Refer to Appendix F if necessary.
2. Place the condenser in its uppermost position. Open the condenser diaphragm fully.
3. With care, rotate the nosepiece so first the 10X and then the 40X objective lens is in line with the body tube. How can you tell when the objective lens is in position? Place the 10x objective in position. Open the lamp diaphragm fully.
4. Turn on the power. Note that you can adjust the lamp voltage until the intensity is comfortable to your eyes.
5. With the objective lens at least 1 centimeter above the stage and looking from the side of the microscope, rotate the coarse adjustment knob through one full turn. About how far does this cause the objective lens to move? Repeat with the fine adjustment knob. How far does the objective lens move? NOTE: The fine adjustment has a limited range through which it operates. Its movement may stop at either end of the range. You can reset the fine adjustment near the middle of its range by adjusting the coarse focus knob.
6. Open the spring-loaded finger of the mechanical stage and insert a prepared slide. Moving the controls will move the slide left and right, and back and forth. Position the slide to center the specimen in the light path.

7. Bring the image of the specimen into focus using first the coarse and then the fine focus adjustment knobs. NOTE: Start with the objective lens close to the slide and move it up away from the slide as you focus.
8. Adjust the ocular lenses for the distance between your eyes so that perfect binocular vision (a single, circular field when viewed with both eyes) is obtained.
9. With your left eye closed, look at the image through the right ocular lens with your right eye and carefully focus the image of the specimen with the fine focus knob. Next, close your right eye and look at the image through the left ocular lens with your left eye. Rotate the diopter adjustment ring at the base of this ocular lens to focus the image of the specimen without adjusting the focus knobs.
10. To focus the light on the specimen:
  - a. Rotate the lamp diaphragm ring counterclockwise to stop down the diaphragm to its minimum opening
  - b. Turn the condenser height adjustment knob in either direction until the image of the lamp diaphragm is sharpest in the field of view. Notify your instructor if the illuminated area bounded by the edge of the diaphragm is not in the center of the field of view.
11. Open the lamp diaphragm until the edges of the diaphragm are just outside of the field of view.
12. To maximize the size of angle  $\alpha$  and thus the value of  $\sin \alpha$ :
  - a. Carefully remove the right ocular and place it beside the microscope.
  - b. Look down the barrel of the ocular tube, and adjust the opening of the condenser diaphragm until the light fills about 70-80% of the back lens of the objective. If the specimen is stained lightly, or almost colorless or transparent, you may improve contrast by further reducing the size of the condenser diaphragm. If you decrease the size of the condenser diaphragm too much, you will reduce resolving power because the value of  $\sin \alpha$  will be reduced.

NOTE: While viewing your specimen, you may wish to make fine adjustments to the contrast of the image. This should be done only by adjusting the size of the opening of the condenser diaphragm or the intensity of the light source. **Do not use the height of the condenser to manipulate contrast as this changes the focus of the light.**

13. Study the specimen.
14. When you change to another "dry" objective:
  - a. Center the object you wish to view.
  - b. Change the next higher objective by rotating the nosepiece.
  - c. Touch up the focus with the fine focus knob. Never use the coarse focus when using high magnification (400X and 1000X)--the proximity of the objective to the slide crack the slide and/or scratch the lens!
  - d. Repeat steps 10 - 13 with the new objective in place.

NOTE: THE 100X OBJECTIVE LENS CAN ONLY BE USED WHEN IT IS IMMERSSED IN A SPECIAL OIL. YOU WILL NOT LIKELY NEED THIS LENS IN BI 163, BUT APPENDIX D PROVIDES INSTRUCTIONS ON ITS USE FOR YOUR REFERENCE.

**Microscope Use Checklist:** Some helpful reminders:

1. Turn on the power.
2. Always begin observations using the 4X or 10X objective lens.
3. Place slide on microscope stage and position specimen in the light path.
4. Bring the image of the specimen into focus. Close your left eye, and bring the specimen into sharp focus with the fine focus knob. Now close your right eye, and bring the specimen into sharp focus using the diopter adjustment ring at the base of the left ocular lens.
5. Close the lamp diaphragm to its minimum opening.
6. Turn the condenser height adjustment knob in either direction until the image of the edge of the lamp diaphragm is defined sharply.
7. Open the lamp diaphragm until the edges of the diaphragm are just outside the field of view.
8. Carefully remove the right ocular, look down the barrel of the ocular tube, and adjust the condenser diaphragm until the light fills about 75% of the back lens of the objective. Replace ocular.

**APPENDIX A**

**Using the Phase-Contrast Microscope**

Phase-contrast microscopy allows the viewing of faint or lightly colored specimens without the use of stains. The phase ring, located just beneath the stage of the microscope, adjusts the light passing through specimen such that contrast between the specimen and the background is enhanced.

Use of the phase-contrast microscope is very similar to use of the standard compound light microscope. However, in order for the phase-contrast properties of the microscope to be realized, the number on the phase ring **must** match the power of the objective being used (10X and 40X in most cases). Be sure to rotate the phase ring to the proper setting each time you change the objective. When using the phase ring, you do not need to make adjustments to the condenser diaphragm.

Do not touch any knobs or controls with which you are unfamiliar--doing so could put the phase ring out of adjustment and the phase-contrast properties of the microscope will be reduced. *If you are not certain of how to use the microscope properly, please ask your instructor for assistance.*

**APPENDIX B**

**Preparation of Wet-Mount Slides**

Make wet preparations by placing a small drop of water or other fluid in the center of a clean slide. Center the object of interest in the drop of liquid. Lower a clean cover slip over the drop gently to avoid trapping air bubbles. If done properly, the moisture of the drop will completely fill the space under the cover slip, with no excess outside that might run onto the microscope stage. If the coverslip appears to be "floating" atop the slide, you have used too much liquid. You will either need to make a new slide or wick some of the liquid away with a Kim-Wipe.

Studying wet preparations should show you that a microscopic preparation, while thin, does have appreciable thickness when observed with the microscope. A sense of the depth of the field is an important feature of study with the microscope. You can see depth of view by using the fine adjustment. As you focus up and down, the two-dimensional images at various levels become integrated in your mind to form a notion of the third dimension of the preparation.

## APPENDIX C Preparation of Fixed Slides

Bacteria in a wet mount are very difficult to see since light tends to pass right through them, rendering them all but invisible. If a phase-contrast microscope is not available, Bacteria must first be 'fixed' to a slide by drying a small smear, heating the smear gently to cause the cells to adhere and staining them with a basic dye to artificially color them.

1. Using a sterile loop, transfer a small amount of bacterial suspension to the slide and spread thinly. Allow the smear to air dry. This may take a few seconds to a few minutes depending on the thickness of the smear. Dispose of the loop in the biohazard container.
2. Once dry, pass the slide carefully through the flame of an alcohol lamp. The slide should just be warm to the touch. If it blackens and begins to smoke, you have heated it too much. The cells should now be permanently attached to the slide and difficult to wash off.
3. Place a drop of methylene blue dye to the smear and wait one minute.
4. Rinse the slide with a squirt bottle of distilled water until no more excess dye rinses off. Blot the slide on a paper towel and you are ready to view it!

## APPENDIX D Use of the Oil Immersion Lens

Since oil has a higher refractive index ( $n$ ) than air, using an oil immersion lens can increase the resolving power of the microscope by decreasing the limit of resolution (as defined by Abbe's equation in "Some Principles of Light Microscopy"). To use the oil immersion lens:

- a. Center the object to be viewed.
- b. Partially rotate the nosepiece.
- c. Place a drop of immersion oil directly over the area on the slide you wish to observe.
- d. Place the oil immersion objective (usually 100x) into position. Check to be sure that the front element of the objective is immersed in the oil. When doing this step, watch carefully from the side of the microscope so that the objective does not contact the slide.
- e. Use the fine focus knob to bring the image into focus. Focus upward so the objective does not contact the slide.
- f. Follow steps 10 – 13 under "Using a Light Microscope with Kohler Illumination" (above).
- g. When your work is complete, **be sure to wipe all oil from the objective with lens paper. Do not get oil on the other objectives.** If you do, wipe it off with lens paper immediately. Oil dried on a lens renders the lens useless.

## APPENDIX E

### Calibration and Use of the Ocular Micrometer

One ocular lens of your microscope should contain a glass disk with fine divisions that may or may not be numbered. This is an *ocular micrometer* that is used to measure objects in the field of view (see Figure 3).

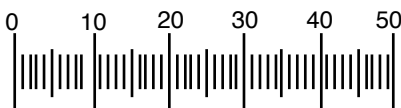


Figure 3. Scale of an ocular micrometer

**NOTE: The numbers on the ocular micrometer DO NOT represent any fixed unit of distance! When you increase magnification, the field of view (i.e., the total area visible through the microscope) decreases, and the ocular micrometer becomes superimposed over a smaller area. As a result, the physical distance represented by each small division (or “ocular unit”) *decreases* with each increase in magnification. For this reason, it is important to know the calibration of the ocular micrometer for the specific microscope you are using!**

The ocular micrometer is calibrated with a *stage micrometer*, a precise scale prepared as a dry mount (Figure 4). Since the units on the stage micrometer are constant, it may be used to “measure” the ocular micrometer when used with the different objective lenses. This is less complicated than it sounds, as you will discover if you work through the following procedure:

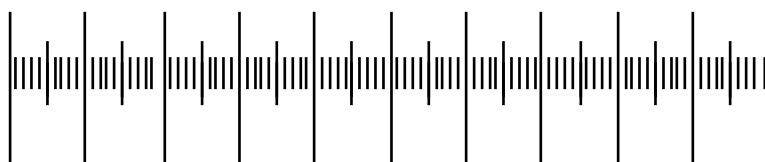


Figure 4. Calibration lines on a stage micrometer. Each division = 0.01 mm

#### Procedure

1. Focus on the calibrations of the stage micrometer using the 10X objective.
2. By rotating the ocular lens containing the ocular micrometer and moving the stage micrometer with the mechanical stage, superimpose the scale of the ocular micrometer over that of the stage micrometer (see Figure 5).
3. Determine the length (in mm) of each division on the ocular micrometer by dividing the length (in mm) covered on the stage micrometer by the number of divisions on the ocular scale. Each small division on the stage micrometer = 0.01 mm. Calculate and record the length of an ocular unit.

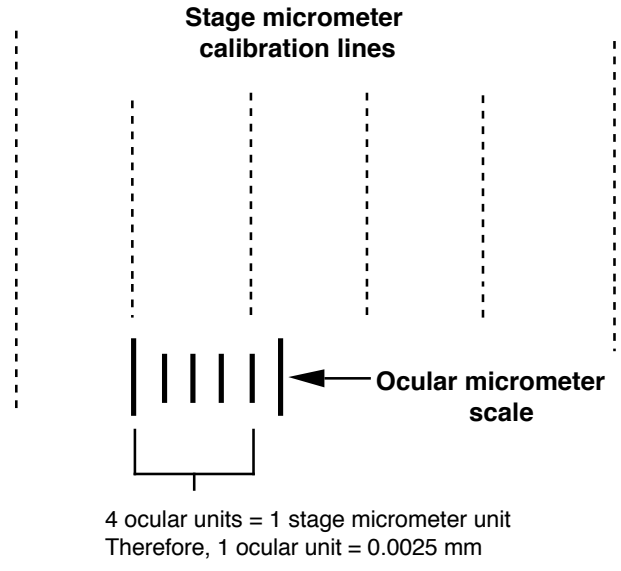


Figure 5. The ocular micrometer scale superimposed over the stage micrometer scale.

4. Carefully change to the 40X objective and repeat part 3 to find the length of each ocular micrometer unit at that magnification.
5. **Do not attempt to focus on the stage micrometer calibrations with the oil-immersion objective.** The impact could break the thick stage micrometer.

The ocular micrometer is a useful tool for measuring microscopic specimens, but the micrometer itself is not typically shown in illustrations or micrographs. For these applications, a *scale bar* should be used (Figure 6). It's helpful to draw the scale bar approximately the same length as the specimen of interest, with the actual distance being determined with the ocular micrometer.

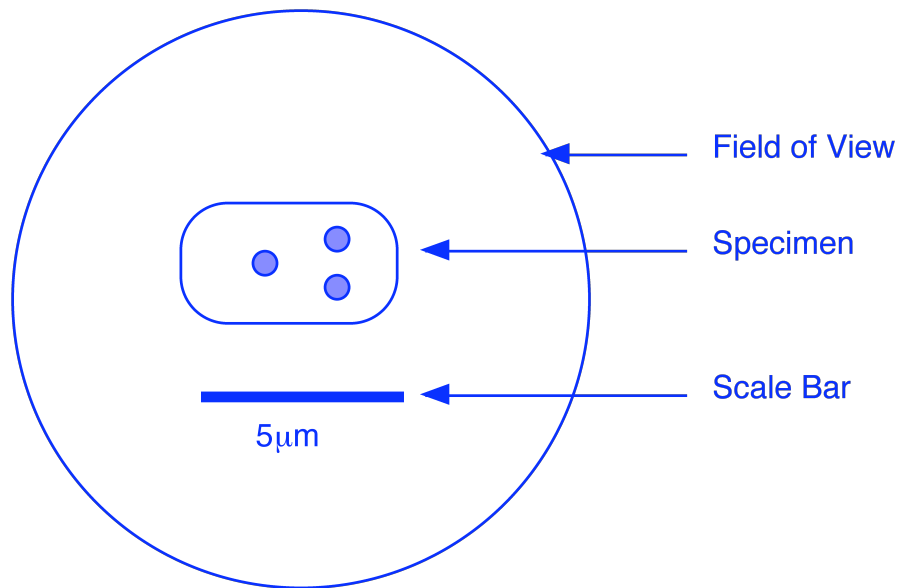


Figure 6. Illustration of a microscopic specimen with a scale bar included.

**APPENDIX F**  
**The Olympus CH-2 Compound Light Microscope**

