CHAPTER 2-1
LABORATORY TECHNIQUES: EQUIPMENT

Janice M. Glime and David H. Wagner

TABLE OF CONTENTS

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Figure 1. Dries Touw at a microscope in the Hattori Botanical Laboratory, Nichinan, Japan, 1983. Photo by Janice Glime.

Lower Plants

Moss and lichen – lower plants,
the higher plant people say;
But if you give them half a chance,
they'll really make your day.
Miniature beauty – ecology too,
enough for your interest forever;
You'll need a scope and some chemicals few,
to unlock their secrets most clever.
So get out there – look around,
learn from the lichen and moss;
Treasure the mysteries of lower plants found,
and you'll never be at a loss.

- Ray Showman

Lab bench Setup

A well stocked lab bench (Figure 1) will save you time and make your work more efficient. Usually this bench will be located near the herbarium specimens for easy checking of your identification and for processing and accessioning specimens (Figure 2).

Figure 2. Working area in the herbarium at the Missouri Botanical Garden. Specimen compactors are on the left and benches with microscopes and other equipment are on the right. Photo by Paul J. Morris through Flickr Creative Commons.

Although making slides seems relatively routine for anyone who has done this often, there are lots of tricks to make it fully effective. Having a set of tools that are in one place is a tremendous time-saver, and it encourages one to

identify that specimen picked up on the way to work rather than tuck it away for later. This can be accomplished easily if you have a workbench reserved for that purpose, but if not, create a sturdy box that holds your needed tools.

This is a **list of supplies** you will probably want to have handy so that you can proceed efficiently:

Desirable Lab Bench Supplies

compound microscope with its own base light source (Figure 5)
dissecting microscope with top mounted light or lights on each side (Figure 8-Figure 9)
blue filters for microscope lights
microscope slides (Figure 33)
coverslips (Figure 31)
single-edged razor blades
microforceps or watchmaker forceps
a curved one and a straight one are helpful (Figure 25)
whetstone
emory board
dropper bottle with narrow tip and water (Figure 29)
ocular micrometer
stage micrometer
lens paper
paper towels
Petri plate(s)
Syracuse watch glass (Figure 3)
hot plate for warming water to soften tissues
jar or beaker of water
slide labels
permanent mounting medium
methylene blue or similar stain

Figure 3. Syracuse watch glass.

Microscopes

As Rockcastle and Barr (1968) pointed out, observing bryophytes requires a "bits made big" technique. Identification of bryophytes usually requires two microscopes, a dissecting/stereo microscope with a zoom (preferred) viewing range of ~3X to 40X and a compound microscope with a viewing range of 40X (or less) to 400X total magnification. Magnification is determined by multiplying the ocular (eyepiece) magnification by that of the objective lens. Higher magnification may be required, depending on your needs.

The dissecting microscope is needed for several purposes. It is usually the first microscope you will want to use to get a clearer view of leaf insertion, paraphyllia, decurrencies, general habit, and other surface features. It is also needed for locating small bryophytes, especially tiny leafy liverworts, from among the dominant bryophytes. It is also needed for locating the fauna. The other important use of this microscope is to guide your hands when you make sections or remove leaves.

The compound microscope is used with microscope slides. It permits you to see cell shape, number of cells wide, borders, costae, and teeth on the leaves. On the sporophyte you can see such details as peristome decorations and imbedded stomata. The compound microscope is also needed for a clear view of your sections of leaves and stems. It will also help you see special structures like gemmae, bulbils, paraphyllia, and reproductive organs.

There is a wide range in quality of microscopes. It is very useful to have trinocular microscopes that can hold a camera (see the two microscopes in Figure 1) or to have a USB connection, or that have a direct image transfer to your computer. Once you have a little experience, you can take pictures that are adequate for identification. This can avoid the need, in some cases, for sending specimens to experts, and it will save them lots of time because you have already made sections and spent the time to get good views of the leaves, insertions, and stem sections. You can also introduce these images on Bryonet <Bryonet-L@mtu.edu> or a web site where you can request help for troublesome species. (Caution: try to keep the total of all image sizes under 2 MB when sending them to an email list such as Bryonet.)

One can usually count on Olympus and Nikon microscopes to be of good quality, but these tend to be rather expensive for a beginner. Tamás Pócs (Bryonet 10 February 2012) reports great satisfaction with the BioLux NV (Figure 5), a compound microscope made by Bresser, Meade Instruments Europe Bmbh & Co. KG, Gutenbergstrasse 2, DE-456414 Rhede/Westf. Germany at a price of about 100 Euro <http://www.astroshop.eu/monocular-microscopes/bresser-microscope-biolux-nv/p,14667>. Its magnification ranges 20-128 X. It is lit by LEDs (with transmission and overhead light) and also has a digital camera ocular. It also has a movable stage and several built in filters, as well as a carrying case for field work. An added advantage is that it has a carrying case, making it more convenient for field work.

Figure 4 outlines the principal parts of a **compound** microscope. Since manufacturers vary, the microscope

available to you may differ somewhat. You will note that some microscopes have one eyepiece (**monocular**; Figure 5), whereas others have two (**binocular**; Figure 9). **Ocular** refers to the eyepiece.

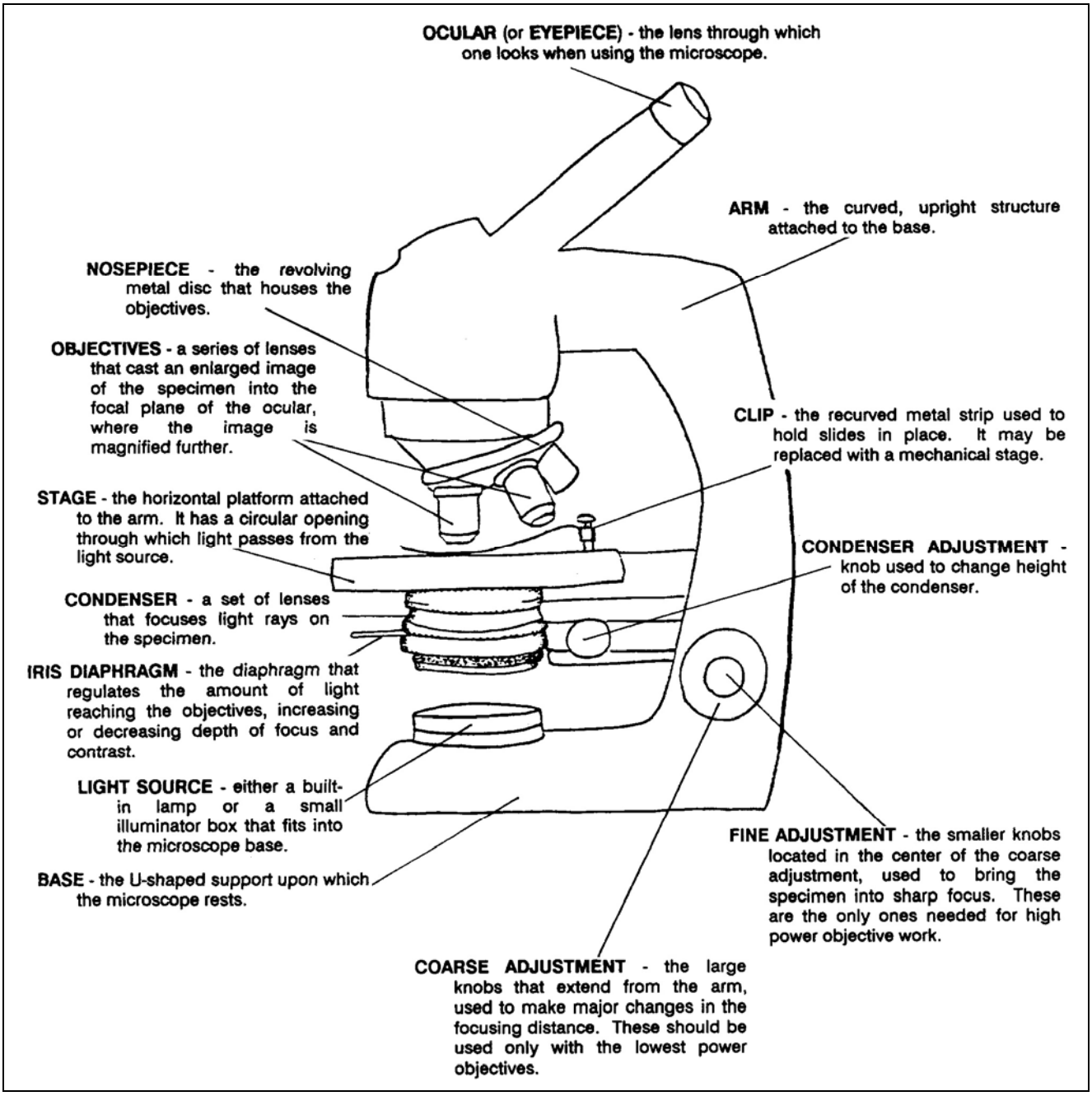

Figure 4. Compound microscope showing parts. Drawing by Janice Glime.

Figure 5. BioLux NV compound microscope, made by Bresser. Photo modified from Bresser website.

The **mechanical stage** (not shown) has numbers in both directions that you can move the slides, so that you can note the coordinates on the numerical scales, then remove the slide. When you replace it you can go easily to the same location.

Your microscope should be **parfocal** (having all lenses adjusted to the same focal distance, making it possible to switch objective lenses with minimal refocusing). That means that when you change from one objective to another, the ocular distance is still correct and the object should still be close to being in focus. When you start using a different microscope, it will most likely be somewhat fuzzy for several reasons. You have increased the magnification and it is easier to see that it is not in perfect focus. The oculars may not all be screwed in completely, changing the focal distances. However, in most cases you can adjust this by using the fine adjustment. Different people have different focal distances, and these change with time. Hence, some initial adjustment is needed, with fine-tuning occasionally.

Parfocal Adjustment

Binocular microscopes may require adjustment so that both oculars focus at the same distance. If there is also a camera tube, this also needs to be adjusted.

Procedure

1. Position the objective turret above the subject.
2. Set both eyepiece diopters to "0."
3. Set the microscope near the middle of its focus range.
4. Focus with the microscope on the lowest magnification setting, using first just one ocular (eyepiece). Use the fine adjustment until the image is clear.
5. Focus with the other ocular by turning the diopter ring on the ocular until the image is clear and sharp. Do not move the fine adjustment. If you can't get the focus clear, put it in the best position, use the fine adjustment, and readjust the other ocular. Repeat until both oculars provide clear focus.
6. Move to the next highest setting and repeat the process.
7. Finally, move to the highest setting and repeat the process. If you will not be using the oil immersion lens, then use the highest magnification below that.
8. Tighten the diopter lock button if there is one and record the setting.
9. Repeat the procedure with the microscope tube.
10. Do this whenever using a microscope that is new to you. As your eyes change, the settings may need adjustment.

Microscope Use

First, it is important that you learn to use the microscope correctly, or you will not be able to see all the things that you should. After you understand how to set up your microscope, make a slide of one of your samples and determine the best way to adjust your microscope.

Adjusting Light and Learning to Focus

1. Start with a **prepared slide**; diatoms are a good choice because of the fine detail of the pores and striations. The diatom *Amphipleura pellucida* is often used. Its striae (lines of pores) have a mean of 0.25 µm distance apart, the theoretical limit of resolution of light optics. If you can see them clearly, it is an indication of a good quality of microscope. If you don't have access to a diatom slide, you can use an onion skin (the thin layer) or other thin, nearly transparent subject in a drop of water with a coverslip.
2. On the slide you can often **locate the organism** by a change in color as you scan, but a more reliable way is to begin by focusing on the edge of the coverslip. This will put your view in the right plane so you can scan the slide.
3. Once you locate the specimen, **focus first with the coarse adjustment**, always starting by focusing upward so you don't run the objective into the slide.
4. Once you have gotten the clearest image possible with coarse adjustment, use the fine adjustment to get and even more distinct image.
5. Using a nearly transparent slide, you can learn to **adjust lighting**. This should be done by adjusting the diaphragm under the condenser. It should **not** be adjusted with the light intensity of the light source because with most microscope lighting systems, lowering the light intensity changes the light quality to the red end. Changing the light intensity by using the rheostat to change the intensity of the light source should only be used to adjust the light for your eye comfort.
6. If the light has a red cast, it can be balanced with a **blue filter** (see Figure 6-Figure 7). This can be especially important for photography. Usually it just requires a blue glass filter over the light or in the condenser housing under the diaphragm, and often one is supplied with the microscope.

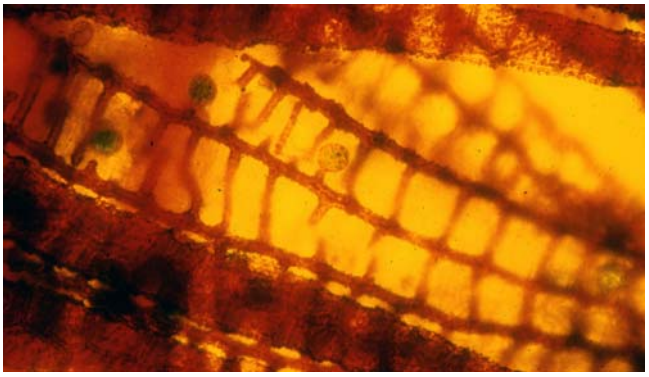

Figure 6. Peristome of ***Fontinalis squamosa*** with tungsten light of microscope and no blue filter. Photo by Janice Glime.

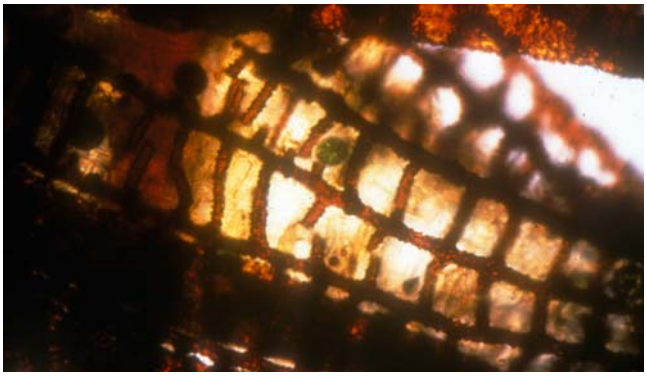

Figure 7. Peristome of ***Fontinalis squamosa*** with blue filter covering tungsten light of microscope. Photo by Janice Glime.

Adjusting the Focus and Ocular Distance

1. If you are using a binocular microscope, you need to adjust the oculars. If you are right-handed, look through the right ocular with your right eye and get the object into clear focus. Even if you are left-eyed, start with the right ocular, but use your left eye. (If you are right-handed, you are likely to be right-eyed.) You might have to shut the other eye to do this.
2. **Always start with the coarse adjustment and focus upward.** It is safest to adjust the objective downward with the coarse adjustment while watching the slide from the side without using the ocular, bringing the objective as close as possible to the slide without touching it. Then be sure you focus in the opposite direction (upward) **slowly** with the coarse adjustment while watching through the microscope. If you are inexperienced, focus on the edge of the coverslip to get into the right plane.
3. Adjust to the best focus using the **fine adjustment**.
4. Then use your left ocular, still using your right eye, and get it into focus, but this time **turn the left ocular** to adjust the focus. This is usually done by turning a ring at the base of the ocular housing, not the ocular itself.
5. If it is impossible to get a clear image this way, you may have to move the right ocular so it is closer to the middle of its range, then refocus with the fine adjustment before focusing the left ocular. Repeat this until you are able to see clearly with both oculars.
6. Use both eyes, and **move the oculars together** and **apart slowly** until you can see through both of them. At first, you might see two images; adjust the distance between the oculars carefully until you can see only one image in 3-D.
7. When the images are clear, observe the dial or **vernier scale** between the oculars to see what number is indicated. (Some microscopes, especially dissecting microscopes, might not have this scale.) Then set each of the two oculars at that number. This adjusts the focal distance because you have changed it when you changed the distance between the oculars.
8. **Write that number** on your lab notebook, or on a piece of tape on the microscope if you are the only user. You can always set any microscope at this number and save much time in trying to adjust the ocular distance. You will probably have to set the number on the vernier scale every time you come to lab if different people use the microscope because they will have different distances between their eyes.
9. **Re-focus** with the coarse and fine adjustment until you have the best single clear image.
10. After you are more comfortable using your binocular microscope, try adjusting the ocular distance again and record the new number if you find a better position.

Adjustments for Glasses

1. When you adjust the microscope to see the image clearly, you are also adjusting the focal distance to work best with your eyes. This will compensate for near-sightedness or far-sightedness. However, it will not adjust for astigmatism. Examine the object on the microscope slide to see if it is clearer with or without your glasses. This will require a new adjustment.
2. If you prefer to wear your glasses, then you need to take precautions to prevent scratching them. You can use a bit of masking tape on two sides of the ocular to provide a soft cushion for your glasses. Some microscopes will be equipped with rubber caps that protect glasses. The rubber or plastic extensions that fit around your eyes generally get in the way when you wear glasses.

Dissecting Microscope

In addition to the compound microscope (the one you will use with slides), you will also be using a **dissecting** microscope (microscope with low stage and long focal distance that permits you to dissect an object while viewing). That gives you a large viewing and working space that permits you to work with larger objects that you can dissect while viewing them.

The principles of the dissecting microscope are the same as those for the compound microscope, but this microscope will always have two oculars, and lighting will usually be from above, giving you reflected light. Some microscopes have a light in the base, but I (Glime) find these uncomfortable to work with because they make the stage high (Figure 8) and I cannot rest my hands on the lab bench.

Figure 8. Dissecting microscope with two foam pads and a cork board to permit moving the stage up and down while still working. Photo by David H. Wagner.

Self-focusing Foam Stage for a Dissecting Microscope

David Wagner offers suggestions for working with a dissecting microscope when your plane of focus keeps changing. This can be a problem when searching for propagules or reproductive structures and when sorting for the interesting invertebrate fauna. When you move one hand to the focusing knob, it is easy to lose track of the object of interest. He has created his own vertically movable stage. A foam pad can help (

There are foot-controlled, electric focusing stages, but these are very expensive. Wagner's solution is to place a stack of foam pads such as artificial sponges (must be soft when dry) on the stage of the dissecting microscope and placing a cork board on top to provide a solid surface (Figure 8). Set the focus of the microscope to the surface of the cork board. The specimen will be above this, so you can focus on any part of a specimen by pressing down on the board with the heels of your hand without setting down your tools. It is also easy to tip a specimen from side to side while keeping a particular point of interest in view and in focus (Figure 9), even with magnification set at the highest level. With this system, one seldom needs to touch the focusing knob after the initial focus. Glime thinks you might want to add sponges, pillows, or a box on each side of the stage so that you have something on which to rest your hands and forearms. They would be steadier that way, and it would be less tiring. Hence, for that reason Glime prefers a microscope that has a low stage. A lower bench or higher seat might help make you more comfortable.

Figure 9. Dissecting microscope showing the ability to tilt a stage with two foam pads and a cork board. Photo by David H. Wagner.

Summary of Microscope Care

1. Always carry the microscope with **two hands**. Do not attempt to carry anything else at the same time.
2. Avoid touching the lenses with your fingers.
3. Always start your examination of a specimen with the **low power objective**. Rotate the nosepiece to a higher objective if greater magnification is desired.
4. **Focus away** from the specimen. Lower the objective only when you are watching from the side.
5. Keep **both eyes open** during microscope use.
6. Remember to keep the **condenser slightly below its highest position** and to adjust the **iris diaphragm** to achieve the best possible image.
7. **Never focus down** with the coarse adjustment when the high power or oil immersion objectives are in place.
8. Use **oil immersion** whenever the numerical aperture marked on an objective is greater than 1.00.
9. Clean the lenses with **lens tissue** and **water** or **saliva** only. Avoid cleaning with powerful solvents like acetone or xylene.
10. When you are done, **rotate the low power objective** into position and **remove the slide**. Turn off the light source and cover the microscope.

Microscope Light Sources

A compound microscope typically has a tungsten light source in its base. This usually causes the image to have a reddish cast, so it is desirable to place a **blue filter** under the condenser or over the light to balance the light. For the dissecting microscope, an even white light makes the best viewing. There are a number of choices for these lights.

For reflected light, I (Glime) have used a dual gooseneck fiber optic light (Figure 10). This provides bright, good quality light and avoids heat of tungsten bulbs, although some of the new LED lights are probably better. Most of them can be focused and the size of the light circle can be adjusted by changing the distance and focus.

Figure 10. Dual gooseneck fiber optic lights. Photo from online advertising.

Regular tungsten microscope lights are hot and are a nuisance to replace when they burn out. **LED lights** last much longer and are cooler. Wagner has marvelled over the amazing products appearing on the market. He has been especially impressed with the intensity of lights now marketed as headlamps for bicyclists. These are intended to be mounted on handlebars or the top of helmets. They are bright enough to serve as truly effective headlights, almost as bright as those for automobiles.

These lights are somewhat expensive, but cost less than a ringlight flash. The 250 lumen Cygolite (Figure 11) is US $130. Some models in cycling stores have up to a 400 lumen rating, costing about a dollar per lumen. Eventually, as production volume increases, costs should come down.

Figure 11. LED light source for lab or field. Photo by David H. Wagner.

These headlamps are compact (10 cm long, 4 cm diameter) with a rechargeable battery that plugs into either a wall receptacle or a computer's USB port. The latter is very useful because it means you can recharge it on a bench without looking for the wall outlet. Once charged, you can move it from dissecting scope to compound scope easily because no wires tether it (Figure 12). The batteries are rated to have a five-hour working time per charge, making the light useful for extended field work. The light can be directed on a colony of bryophytes in a dark forest. Having a steady light source, an image can be composed in a way not possible with a flash.

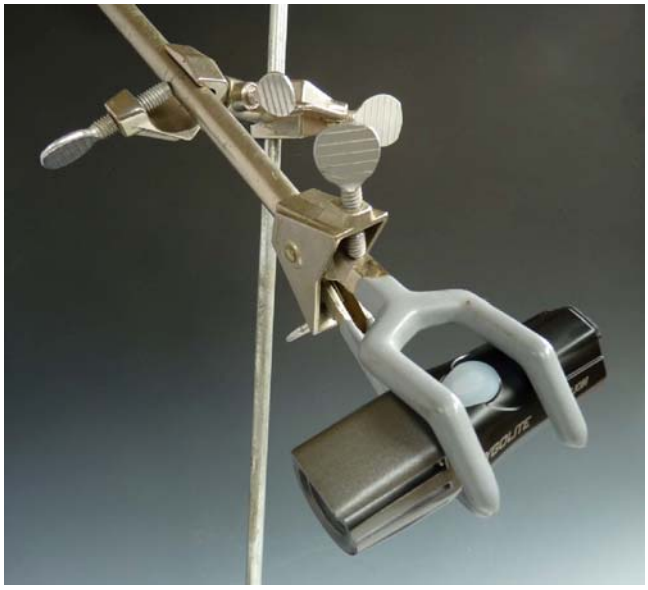

Figure 12. LED light source mounted for use with a microscope. Photo by David H. Wagner.

Zander (2006) suggests using a unit with three 1-watt Luxeonwarm-white LED's, which he has attached to his dissecting microscope with duck tape (Figure 13-Figure 16).

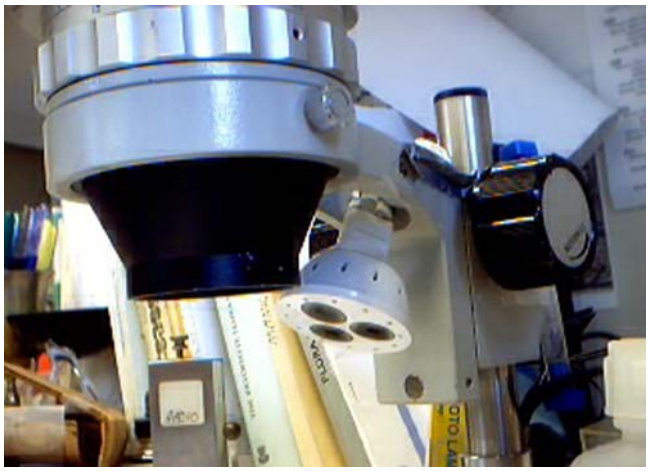

Figure 13. Triple 1-watt Luxeonwarm-white LED unit mounted on dissecting microscope with duck tape. Photo by Richard Zander.

When the LED light is bright enough to be mounted some distance from an object, the light doesn't cause the glare of fiber optic sources, yet is as cool as fiber optic

sources. It is useful in adding **reflected light** to images made with a compound microscope, thanks to stacking software. Compare the two images of ***Jungermannia atrovirens*** androecia (Figure 14), one with transmitted light and the second supplemented by reflected light from an LED headlamp.

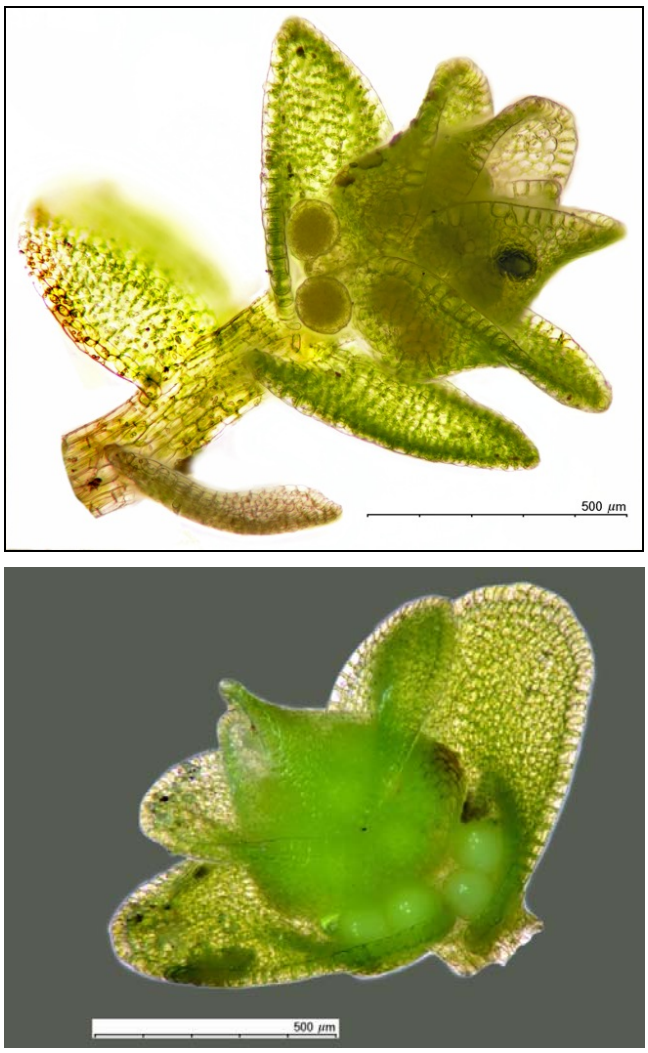

Figure 14. Comparison of light sources for microscopy. Note pasted-in scalebars. **Upper:** transmitted light. **Lower:** transmitted + reflected light. Photos by David H. Wagner.

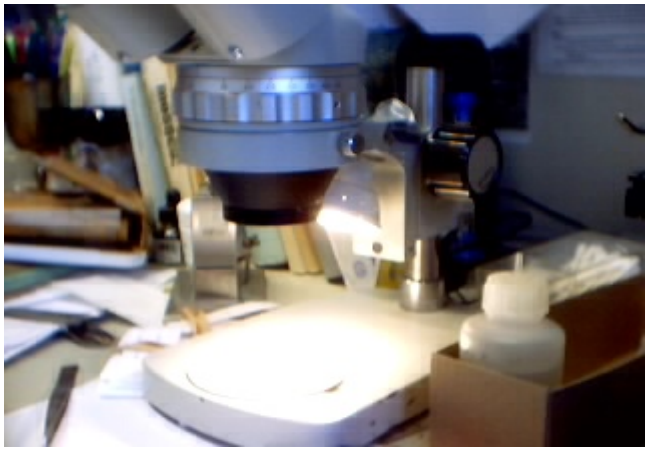

Figure 15. This unit provides an even, warm white light spread over the base of the microscope. Photo by Richard Zander.

Figure 16. The three items needed to provide this LED light system. Photo by Richard Zander.

Three items are needed (Figure 16):

W-15-12 UpLight Power Supply
MR16/MR11 Socket - GX5.3/G40
MR16-WLX3 Warm White LED bulb - Medium 30°

These are available for about US $51 from:

Super Bright LEDs, Inc.
100 Washington St.
Florissant, MO 63031
314-972-6200
<http://www.superbrightleds.com/cgi-bin/store/commerce.cgi?product=MR16>

To set up your light, cut the connector off the end of the line that comes from the transformer. Stuff the copper wires into the holes in the base of the socket, and affix them there with a little duck tape. Plug the transformer into a multi-plug extension cord and use the on-off switch of the extension cord to turn the lamp on and off. For more concentrated light, a narrow-beam 10° bulb is also available.

Differential Interference

Interference microscopy was somewhat popular in the decades from 1940 to 1970. But its complicated design and use caused it to fall into disuse as better microscopes were developed. Its basic principle was to shine two separate beams of light, providing much greater lateral separation than that used in phase contrast microscopy. Gabrys (1978) used it to determine the refractive index of the cell wall of the moss ***Funaria hygrometrica***, demonstrating that for both ***F. hygrometrica*** and the tracheophyte *Lemna trisulca*, the mean refractive index was in the range of 1.41-1.42.

DIC microscopy is one possible way to improve the images. DIC stands for differential interference contrast microscopy and is also known as **Nomarski interference contrast** (NIC) or Nomarski microscopy. This technique enhances contrast in unstained, transparent biological materials. It is the lighting scheme that produces the image, similar to that of phase contrast microscopy, but without producing the diffraction halo that detracts from the latter. This technique has been used for many of the images in the illustrated dictionary produced by Bill and Nancy Malcolm (2006) and in the book *California Mosses* by Malcolm *et al.* (2009).

Ha'penny Optics

Bill and Nancy Malcolm (Bryonet 18 August 2012) have created beautiful images in their well-known *Mosses and Other Bryophytes, an Illustrated Glossary* (second edition). They suggest that to be able to see the delicate details of bryophytes you need differential interference optics. This permits even a transparent specimen to cast a shadow, making it visible (Figure 17). Cheaper microscopes do not have this system, and microscopes from North America and Europe are much more expensive. To solve this cost problem, the Malcolm's recommend a technique called **ha'penny optics**.

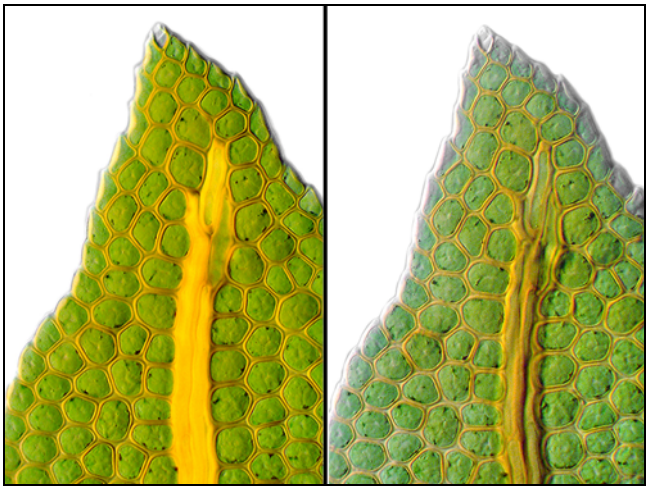

Figure 17. ***Fissidens pusillus*** showing differences in detail in light microscopy (**left**) and **ha'penny microscopy** (**right**). Photos by Bill and Nancy Malcolm.

Ha'penny optics cost only about half a cent, hence the name. The Malcolm's suggest placing a whole mount of a moss or liverwort leaf on your microscope and focusing it under the 40X objective. They slide a memo-sized piece of opaque paper across the underside of the condenser lens. When the edge of he paper reaches the midpoint of the condenser lens, the specimen will appear to be lighted from only one side, with the other side appearing to be in a strong shadow. This creates a 3-d look, revealing the structure of the leaf in excellent detail. This technique, if effective on your microscope, is especially useful for leaf margins, cell walls, papillae, and other textural surfaces. Although this technique works better on some microscopes, it will at least improve the effects on most microscopes. If sliding the paper across the condenser doesn't work, then try sliding it across the light source in the base. The effect is usually best with the diaphragm wide open, but experiment with closing it down slightly. Note that some microscopes have a diaphragm on the condenser and another on the light source.

If this technique works for you, you can make a more permanent **ha'penny** shadow source that is easier to use:

1. **Measure** the diameter of the condenser lens of the microscope (or light source if it works better).
2. Draw a **circle** of the same diameter onto a piece of stiff black paper or stiff plastic (Figure 18).
3. Inside that circle, draw a **second circle** that has a diameter 20 mm less, *i.e.*, making a doughnut 10 mm thick.
4. If the underside of the condenser is curved where it attaches to its mount, a circular doughnut won't lie flat. That can sometimes be solved by making the shape be **D** instead of **O** (Figure 18).

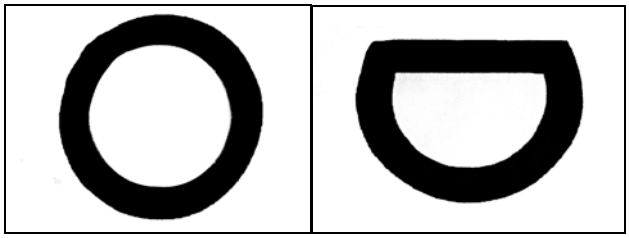

Figure 18. Ha'penny differential interference paper of Malcolm and Malcolm. **Left:** O-shaped interference for flat lenses. **Right:** D-shaped interference that may work better on curved lenses. Drawing by Janice Glime.

5. Cut the **O** or **D** using a sharp utility knife or single-edged razor blade.
6. Make a movable **lever** (Figure 19), using the same plastic or black paper, roughly 160 x 20 mm that will partially block the light reaching your specimen.

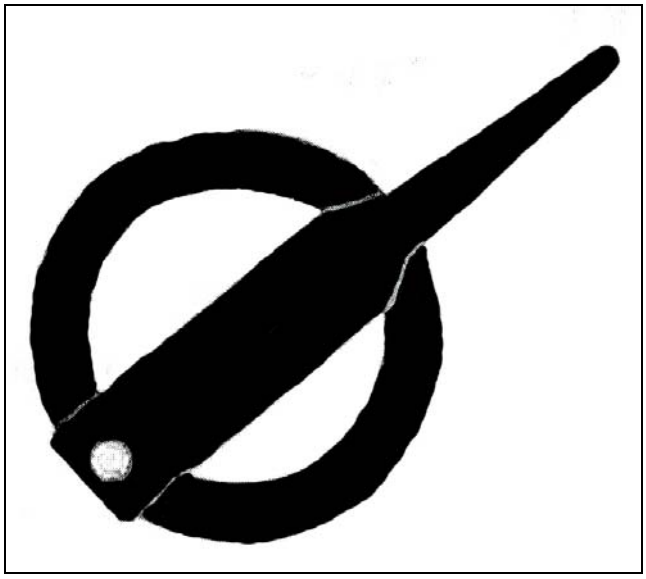

Figure 19. Ha'penny differential interference paper of Malcolm and Malcolm with lever attached. Drawing by Janice Glime.

7. Use a small **eyelet** to hinge the wide end of the lever to the rim of the doughnut (**O** or **D**).
8. Place the apparatus on the light or condenser with the lever on the upper side of the doughnut so that the edge of the lever is approximately at the center of the doughnut when in use, but it must be possible to pull the **lever** completely out of the light path when you don't need its interference. If you are right-handed, you will probably want the lever to be pointing toward the right.
9. **Tape** the doughnut firmly to the condenser or light with the lever on the upper side of the doughnut. Tape the doughnut in three places, making sure the tape does not interfere with the movement of the lever.

Polarized Light

Polarized light can sometimes make certain structures more visible (Amann 1923, 1931; Kolvoort 1966; Nordhorn-Richter 1988). It requires a special microscope with the right optical system and filters.

Plane polarized light can be used to see lignin and lignin-like compounds in vascular tissue and various other tissues where crystalline structure is suitable. Brilliant colors result from the lignin crystals in the cell walls, where the crystals bend the light rays to give the effect you see.

In bryophytes, this might be a tool to find lignin-like compounds in cell walls. The effect is much like a psychedelic panorama. In tracheophytes the lignin occurs between cells such as parenchyma cells and in the secondary walls of sclerenchyma cells. You can see these bright colors in the xylem, but the phloem and cortex cells will be pale or even disappear under plane polarized light. These bright colors are only seen easily in the cross sections of the stem (Figure 20). Certain dyes might also make tissues become visible in plant polarized light.

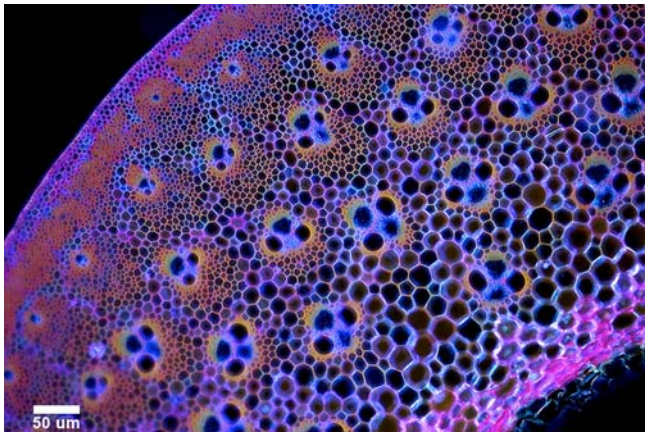

Figure 20. *Fargesia* sp. (bamboo) stem cross section in polarized light. Photo by Eckhard Völcker through Flickr.

Polarized light can tell us about the structure of bryophyte cells. That structure cannot be seen directly, but the presence and direction of crystalline structures can be determined by their response to polarized light (Taylor 1959). They can be seen only when examined in a direction perpendicular to their length, becoming dark (invisible) when viewed in the same direction as their length. For example, the crystals can be seen as brilliant colors in xylem when the stem is viewed in cross section, but not when viewed in longitudinal section.

Leaf Borders and Costae

Adams (2009) has devised a cheap Polaroid system for observing the border and costa of moss leaves. For example, in ***Fissidens***, it makes it easier to see if the costa joins the border at the tip of the leaf. In this case, and others, the moss cell walls have a regular crystalline nature with the cellulose fibers stacked so tightly and in precisely oriented parallel arrays that they rotate plane polarized light. But when the fibers are dispersed in an amorphous hemicellulose matrix, this effect is minimized.

Adams (2009) recommends making **two Polaroid lenses** from a pair of cheap Polaroid sunglasses. These can be cut to fit the filter holder just beneath the substage condenser and to fit the eyepiece lens. If the eyepiece lens is cut larger than the lens, it can be taped on its edges to the ocular. The ocular can then be rotated to block the light (when the gel alignment of the two lenses is perpendicular) and only the properly aligned cell walls will be visible, *i.e.* the border and costa.

Fluorescence

Fluorescence is most widely understood in mosses in chlorophyll fluorescence (Figure 21-Figure 22) (*e.g.* Shi *et al*. 1992; Deltoro *et al*. 1998; Proctor & Smirnoff 2000; Smith 2002; Heber *et al*. 2006; de Carvalho *et al*. 2011). **Fluorescence** is the emission of light from a substance while it is irradiated by light energy (Nordhorn-Richter 1988). Such cell parts as carotenoids, chlorophyll, and phenolic compounds have the necessary conjugated double bonds to cause fluorescence. In addition to these familiar sources of fluorescence, other fluorescing substances include proteins, flavonoids, oils, and waxes. Fluorochromes can combine with specific cell compounds, making it possible to see very small structures with a microscope because these combinations produce secondary fluorescence.

Figure 21. ***Fontinalis squamosa*** protonema using tungsten microscope light with blue filter. Photo by Janice Glime.

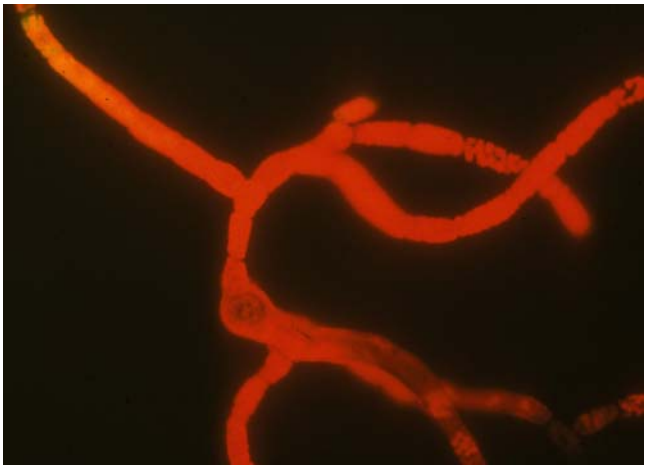

Figure 22. ***Fontinalis squamosa*** protonema using fluorescence microscopy and UV light source. Note that the chlorophyll fluoresces red. Photo by Janice Glime.

To view fluorescence, a fluorescence microscope is needed, equipped with a UV light source. An excitation filter (BP 350-410 or BP 450-490) is needed to absorb all wavelengths except the violet light at 350-410 nm or blue

light at 450-490 nm wavelengths (Nordhorn-Richter 1988). The microscope has a light-splitting mirror, which combined with the filtering system provides a light that permits observation and photography of structures that meet the chemical requirements.

When electrons absorb energy, they are elevated to a higher energy level. This is an unstable state that lasts for only about 10^{-15} seconds, during which they emit light as fluorescence (Nordhorn-Richter 1988). For fluorescence to be visible, the substance must be illuminated with UV light or blue light and the fluorescence is seen as yellow, orange, or red. The reaction requires that the energy content of the radiation source corresponds with the energy conditions of the electrons of the excited substance, requiring a broad spectrum of light energy so that the needed wavelength is present.

Details on applications of fluorescence microscopy are in Chapter 2-2 of this volume.

Dark Field Microscopy

Dark field microscopy takes advantage of the differences in light patterns between the specimen and the blank portion of the slide (Figure 23). The specimen will scatter light, whereas the area with no specimen will transmit the light with no scatter. Dark field microscopy uses ? to exclude the part of the image that does not scatter light. That is, it blocks light from anywhere but the specimen. The result is a greater contrast for the specimen.

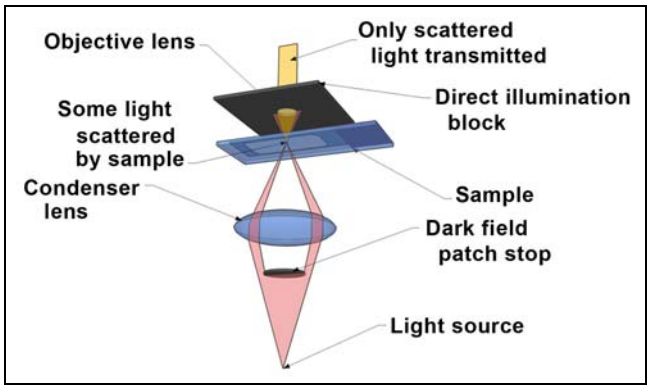

Figure 23. Dark field microscopy setup. Illumination modified from Wikimedia Creative Commons.

Phase Contrast Microscopy

The technique of phase contrast microscopy converts phase shifts by the light passing through a somewhat transparent specimen to make changes in the brightness of the image reaching the eye (or camera) (Figure 24).

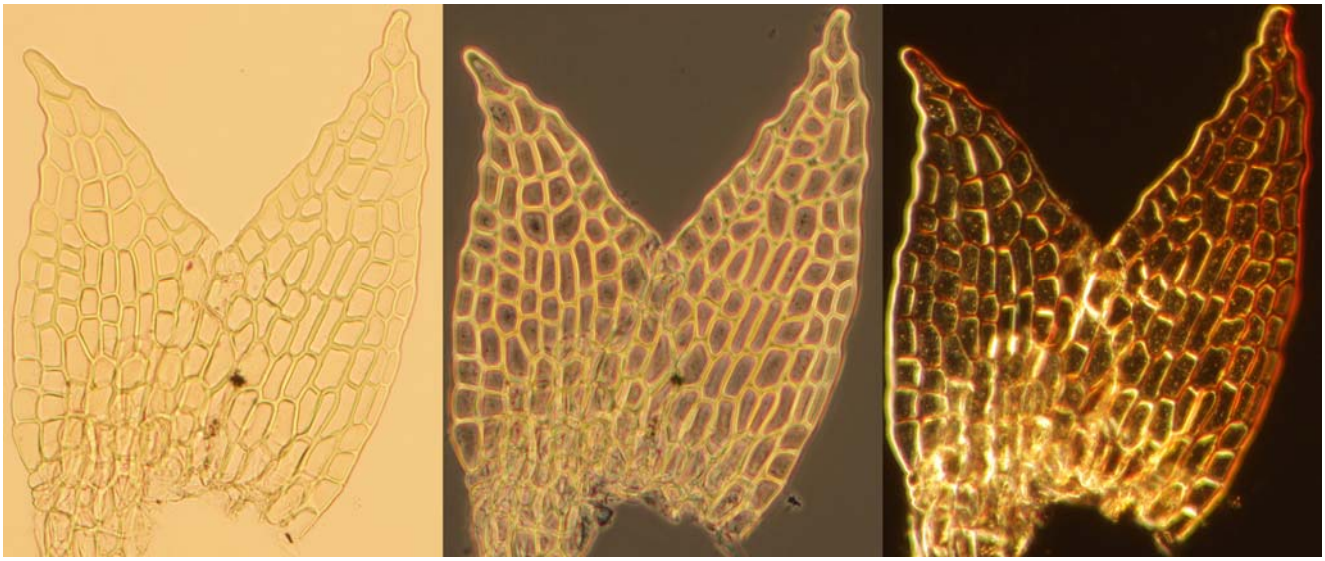

Figure 24. Comparison of microscopical techniques of ***Anthelia juratzkana***. **Left:** light microscopy. **Middle:** phase contrast microscopy. **Right:** dark field microscopy.

Small Equipment

Microforceps

Examining a bryophyte in the lab usually begins with a dissecting microscope and a pair of forceps. And that begins an adventure!

Forceps permit one to widen the spaces between the plants, sometimes revealing an interesting arthropod or gastropod fauna. Rhizoids, gemmae, and other propagules become visible. And it is possible to grasp a single leaf and remove it. **Microforceps** are essential for removing leaves or teasing out tiny liverworts. Standard lab forceps simply are too big.

Do you have leg scars because your forceps fell from you hands and landed point down (Bill Buck, David H. Wagner, Bryonet 5 January 2012)? Some are so expensive that the risk of scars is preferable to buying a new pair. The microforceps, also known as watchmaker forceps, seem to range in price from US $14.95 for a set of 5 on Amazon to US $295.00 from an electron microscopy supply company. But why so costly? Bryophyte parts are tiny. Ordinary lab forceps are much too broad to clasp a single leaf. Hence, one needs those fine-pointed forceps used by jewelers and watchmakers (Figure 25). And those hurt both your vulnerable legs and your pocketbook.

Figure 25. Typical steel microforceps used for work with bryophytes. Photo by Janice Glime.

Prices of US $20-40 are more common for microforceps like the ones in Figure 25. Carolina Biological Supply has extra fine microforceps for US $10.05 (like those in Figure 26-Figure 27). Terry McIntosh (Bryonet 13 May 2010) has found durable steel needle-nose forceps at Canadian $1.00 each that work just as well as the expensive ones. These are available at a liquidator place (Midland Liquidators) in Vancouver, Canada. I (Glime) also have found that the cheap ones work as well as the expensive ones, and last as long. The only problem with some forceps, especially for larger male hands, is that these tend to be a bit shorter, giving large hands less control. I (Glime) have used these in botany and bryology labs – they withstood student use.

Figure 26. These forceps are inexpensive but work well. Photo by Janice Glime.

Figure 27. Forceps with points (and people) protected by a short piece of tubing. Photo by Janice Glime.

Bryonetters (January 2012) discussed the pros and cons of a variety of forceps. Wagner recommends BioQuip #4524 for around $20. Charles Epsey (Bryonet 3 January 2012) and Guy Brassard (Bryonet 3 January 2012) recommend **Dumont** (style 3) in Switzerland for superfine, precisely aligned tips at a reasonable price. I have to agree with the assessment of affordable good quality of the Dumont forceps. They are available at <http://www.finescience.com/Special-Pages/Products.aspx?ProductId=306&CategoryId=29> or <http://www.dumonttweezers.com/>. Richard Zander (Bryonet 3 January 2012) recommends forceps from Micro-Mark. Carl Wishner (Bryonet 3 January 2012) finds ultra-fine and microfine forceps at Fry's Electronics (ER0P5SA stainless, anti magnetic, anti-acid).

Some forceps are "stiff." There is little that can be done, and don't spread your good forceps because they too will become stiff. Once you find a brand you like, stick with it.

For students, cheaper versions of microforceps are sufficient for short-term use, especially if you are supplying them for their use. I recommend keeping one pair of curved microforceps (Figure 25). There are some leaves that are easier to grab with these, and they are good for holding a stem firm with one hand while the other does the pulling. They are also good for removing debris or small arthropods from among the bryophyte stems.

René Schumacker (Bryonet 9 July 2008) suggests also searching for watchmaker tweezers (numbers 4 or 5). Richard Zander and others (Bryonet 8 July 2008) suggest Micromark <http://www.micromark.com/> as a source for inexpensive fine-tipped forceps.

Forceps will last longer if they are kept in a case. Some come in a clear plastic case with a foam sponge to hold them in place so the points don't get knocked around and damaged, but the hinges break easily and you may want a more permanent solution. Cairns (2013) suggests using a pocket reading glasses case for storage (Figure 28). The case should be cushioned at the end where the points go to avoid damaging them. You can cut the tip from a plastic pipette to protect the tips.

Figure 28. Forceps case using a pocket reading glasses case. Note that the upper pair is protected by the tip cut from a pipette. Photo by David Meagher.

Forceps Repair

The inevitable microforceps dropping is likely to require some repair work. They seem to land naturally on their points, blunting and bending them. I have been able to do reasonable straightening on a pair of those cheap student forceps used for ordinary animal dissection. I find that using a curved pair works best – you can stroke the ends of your microforceps like you are milking a cow.

Keep a fine-grain whetstone for needed sharpening or shortening of one side when they are unequal. One approach for restoring the fineness of the tips is to use a

dissecting microscope and whetstone to file the points down to a finer tip (Brian Heitz, Bryonet 3 January 2012). Carl Wishner (Bryonet 3 January 2012) recommends use of a dissecting scope and microfine or fine file "Washita stone." Wagner claims he can upgrade many poorer quality forceps by working with a fine (or extra fine) diamond jeweler's file under a dissecting microscope, but they do not reach the quality of a good pair of Swiss jeweler forceps. Claudio Delgadillo Moya (Bryonet 5 January 2012) suggests putting a final touch on the forceps with fine-grained sandpaper, working under the dissecting microscope. You can also use fine-grained sandpaper to file them, but a whetstone, albeit a more expensive initial investment, is ultimately cheaper because it is usable for a long time.

Ken Kellman (Bryonet 3 January 2012)#has some success in bending forceps back into shape by grabbing both arms of the forceps in fine needle-nose pliers. He then uses a diamond double-sided nail file (emery boards are usually too coarse, but better than nothing), and lightly pinches the forceps around the file and files the inside edges of the forceps (Bryonet 20 March 2023). This tends to get rid of the uneven grip of bent forceps. This even filing of both points simultaneously eliminates the little gaps that prevent you from gripping moss leaves and can even improve cheaper new forceps. File until you can no longer see any gaps under the dissecting microscope.

If your forceps have become bowed, you might want to wrap them in a soft cloth or paper towel to prevent scratches and force them together with pliers. For those broken tips, if possible, file down the longer tip to match the shorter length and file the sides to make the narrow width you need. A whetstone might be needed if the break is very far from the end. Ordinary emery boards will work also, but need to be replaced often.

Although dropped forceps with damaged points can be filed to make them sharp again, if you need to do this often, you soon get to wider and thicker portions that cannot be sharpened adequately. To reduce breakage and bending, store them with a tip protector such as seen in Figure 7.

Microdissecting Needles

Microdissecting tools are often needed with tiny leaves (Deguchi & Matsui 1987), such as those of ***Cephaloziella*** or ***Ephemerum***. I (Glime) learned to make a fine tool from entomologist friends. Such a needle can be made by cutting off the head end of an insect pin, leaving about 2-3 cm at the point end, and embedding the dull end into the end of a wooden matchstick. Cut off the flame end of the matchstick with a pair of pliers and you have a microdissecting needle. A slightly larger point can be made with a sewing needle embedded into the handle in place of the needle part of a cheap lab dissecting needle.

Dropper Bottles

Dropper bottles are used to make slides, but can also be used to moisten a portion of the bryophyte sample without making the entire sample wet or removing a portion of the clump for soaking. This also removes the need to dry the sample again if the moisture is confined to a small portion.

Needle Dropper Bottle

A small, ordinary dropper bottle, even one from the pharmacy, will work for most purposes, but sometimes a smaller drop is desirable, especially if you need to add only a tiny bit of water to the edge of a slide. Wagner (Bryonet 11 May 2010) shared a contribution from one of his students who brought a needle dropper bottle to class (Figure 29). These cost US $3-4 and are made by Gaunt Industries. They are available directly from that company <http://www.gauntindustries.com/2_Ounce_Products-HYPO_25.html> or from plastics fabrication stores such as Tap Plastics <http://www.tapplastics.com/shop/product.php?pid=409&> or art supply stores. Those with 23 gauge needles are best. An added advantage is that they don't spill if knocked over.

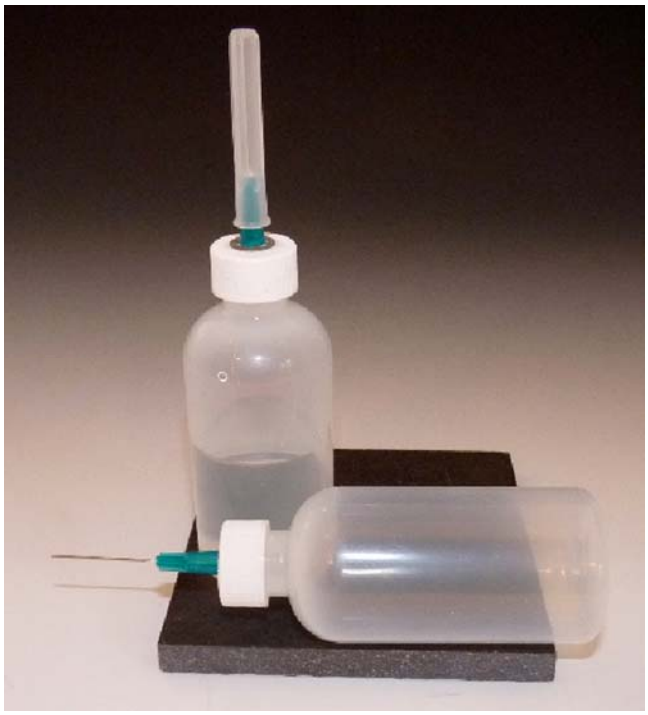

Figure 29. Needle dropper bottle for wetting mosses or adding water to a slide. Photo by David H. Wagner.

Slides

Slides are pretty standard, so little need be said. Occasionally a **depression slide** may be useful so that the bryopFigure 27hyte can maintain its 3-d relationships. Depression slides may be especially useful for observing the fauna in association with the bryophyte.

Coverslips

Plastic or glass? Plastic is cheap and throw away. But plastic is an oil product, so you are using a non-renewable energy resource. That said, there are other issues that are more important for your viewing pleasure. Glass is less likely to get scratched and has different adhesion properties with water. But thin glass coverslips get dirty and break easily. To clean them, get them wet and lay them on a paper towel. Then rub another part or different paper towel over them while they lie flat on a table. Do not try to wipe them between your fingers. They will break most of the time.

Housing for Coverslips

If you have lots of things on your lab bench, It is easy to overturn the coverslips. Zander (1993) suggests gluing the coverslip box to the base of the microscope or to a tray that holds your tools.

Wagner (Bryonet 2011) prefers to keep his coverslips ready for use on a small **polyurethane foam pad** (Figure 30). A small piece, 5 X 10 cm and .5 to 1 cm thick is all that is needed for two or three coverslips. This arrangement makes them easy to pick up by pressing thumb and finger into the foam on either side of the coverslip (Figure 31). The coverslip is then transferred to forceps for careful placement on the slide. But coverslips can be hard to control with forceps because the glass coverslip is slippery. I prefer to hold the coverslip gently by the edges, then with one edge touching the slide, I lower the opposite edge onto the slide with a dissecting needle (See Chapter 2-2 for diagram).

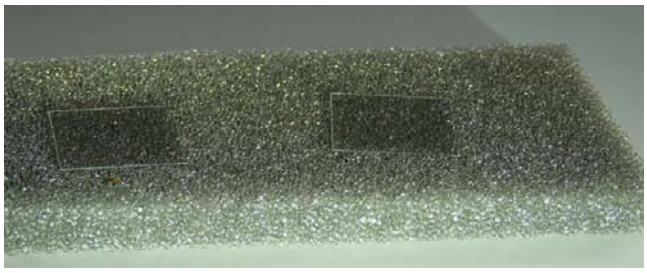

Figure 30. Polyurethane foam for holding microscope slides. Photo by David H. Wagner.

Figure 31. Demonstration of picking up a coverslip from a foam pad. Photo by David H. Wagner.

Standard microscope slides can be placed on a foam pad the same way as coverslips but Wagner prefers to keep them flat on the lab bench. This prevents the drop of water for mounting leaves from getting tipped off. After placing the coverslip on the mount, slip the slide to the edge of the bench to put it on the compound microscope stage. Otherwise, as often as not, the water drop is not added to the slide until it is on the stage of the dissecting microscope and dissections are complete.

Coverslips and Slides in Box

Wagner (Bryonet 2011) keeps his foam pad with coverslips in a wooden box (Figure 32), so that when he's away from the bench for a period of time he can close the lid to keep dust off the coverslips. Cut out a place at one end to store a small stock of coverslips and stack a similar stock of slides at the other end of the box (Figure 33). A reticule for measuring or counting is tucked into a slot in the foam. A fresh razor blade can be stored along the top edge. Wagner's box is not just any old cigar box, it is a box made by a craftsman. It is a way to have an article of beauty on the lab bench, something that can be important to a scientist with an active aesthetic sense.

Figure 32. Storage box used by David H. Wagner for storing slides and coverslips. Photo by David H. Wagner.

Figure 33. Open storage box used by David H. Wagner for storing slides and coverslips. Photo by David H. Wagner.

Other Useful Tools

Annie Martin (Bryonet 13 May 2010) gives us the perspective of an amateur beginner. She tells us that as she entered the world of bryology and started her own closer inspection of mosses, she discovered a couple of "creative"

tools of benefit. First, Paul Davison suggested a **syringe** as a sharp tool for cutting small fragments. She found a package for about $5 available at a local drug store (but she had to ask the pharmacist to retrieve them from behind the counter).

An idea she claims as her own hillbilly thinking – use a **camping headlight** (cut off the head strap) for a portable light source for microscopes. These head lamps are super bright with several settings, including a spot light. They have a foam backing that keeps them from sliding around on the surface. They actually provide better light than the battery lamps with goosenecks and range in price from US $5-$25. The lamps are available in outdoor stores or camping sections of superstores.

Being the daughter of a dentist, Martin found several dental tools with sharp points that work. When you are not affiliated with a university or have access to a research lab, it becomes necessary to find all kinds of creative, cheap solutions.

Photomicrography

Photography has been improving rapidly with the onset of digital cameras (Frahm 2000a, b, 2002) and stacking. But taking pictures through the microscope is still somewhat challenging – and can be expensive to set up. Some suggestions will be discussed here, but more detail will follow later in a chapter on photography in this volume.

There are several ways to get images of microscopic structures: cameras, digital connections to a computer, and scanners.

Scanners

Scanners can sometimes make relatively good images of flat objects and can therefore be used to show larger leaves or whole plants (Figure 34-Figure 35). The only magnification you can get is digital, but it is sometimes adequate when you want to get a quick image at home.

Figure 34. ***Plagiomnium*** sp. image (drying) from an Epson V500 scanner. Photo by Janice Glime.

Figure 35. ***Fissidens*** sp. image from an Epson V500 scanner. Photo by Janice Glime.

Experimentation with a scanner demonstrated that one can get reflected images that way, and in the absence of a camera that is able to get close to the subject, it can at least provide a habit image of some bryophytes. But the Epson V500, which is a good flatbed scanner that does an excellent job of transforming 35 mm slides into digital images, is unable to recognize the mosses as an image when using the transmitted light as one would for an image of a 35 mm slide (positive film). I (Glime) was unable to cajole the scanner into making a transmitted light scan of ***Fissidens*** or ***Plagiomnium***. It might be useful for making images of microscope slides of a sufficiently large specimen, or even of larger samples of bryophytes directly on the glass, but that remains to be demonstrated.

Malcolm and Malcolm (2006) describe using a scanner for photographing bryophytes. They recommend using at least **2400 dpi** resolution and then enhancing the image in Photoshop. The white inner lid of the scanner can be replaced with an upside-down tray at least 5 cm deep and spray painted flat black (or some other desirable color), with the spray painting avoiding the brush strokes. Avoid a white background because it can present uneven glares. For light-colored capsules and some bryophytes, use a grey background instead of black to avoid too great a contrast.

The specimen should be well **hydrated**, but not dripping. If condensation occurs, you can warm the platen (glass) of the scanner with a hair dryer. Clean the platen with a cotton cloth between scans to remove dirt, being careful not to scratch it with adhering sand.

Place the specimen upside down on the platen, using one of the upper corners. This will give the appearance of light coming from one side as it would in nature. You might want to test the scanner with a uniform, highly detailed color pattern that covers the platen. That will tell you if the scanner has "sour" areas that do not focus well or render colors well.

Choose **reflective** mode on the scanner and select an area slightly larger than the specimen. Use 100% scale (**original**). **Turn off** sharpening and compression and do all manipulating in Photoshop. The gamma should be set at 1.8.

To improve the image clarity and contrast in Photoshop, experiment using LEVELS (and select PREVIEW). Adjust colors as needed and sharpen last. Keep your original completed image as a TIFF or PICT file and make a copy before changing to jpg or other lower resolution format. The 2400 dpi resolution will permit you to crop your image while retaining sufficient detail.

Cameras

Michael Lüth (pers. comm.) recommended a Nikon Coolpix 990. This camera can be placed directly onto the ocular of the microscope. A wide field ocular is best to avoid having a circular image. This camera lens is the right size to fit well and exclude stray light. You can look directly at the camera screen to see the microscope image. The camera can be used on both compound and dissecting microscopes, as well as being a general-use camera for close-ups or scenery shots. For both types of microscopes, a third lens dedicated to the camera works best because it is level. It is often necessary to do some adjusting to make its focal plane match that of the other two oculars. But since it

is relatively easy to see the image with the camera, focusing can be done using the camera view.

Many microscopes now are being sold with a digital ocular that connects directly with the computer. Usually you can view the image on the computer screen, making focusing easy. Some of these have excellent quality, but some have very poor quality, being very pixilated due to low resolution. Don't order one unless you can see what it is capable of doing.

If you already have a camera tube on your microscope, or even the right diameter normal ocular, you can make the connection to your computer with an inexpensive attachment such as the one by GeckOOptics (Figure 36-Figure 37) for Australian $125. The included software package allows you to view, save, and edit images.

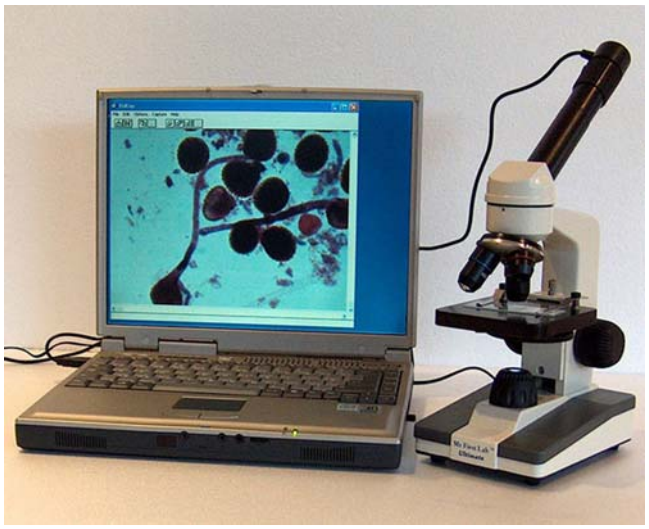

Figure 36. Computer screen with USB hookup to microscope. Photo by GeckOOptical. PERMISSION PENDING

Figure 37. USB attachment for microscope from GeckOOptical. Photo by GeckOOptical. PERMISSION PENDING.

Scalebar

For demonstrations of cellular structures or spores, size is important. Hence, providing a scale with the image is important. Rod Seppelt (Bryonet 14 April 2010) suggested taking a picture of a stage micrometer (special microscope slide with a microscopic scale). This should be done for all magnifications that you are likely to use. The image could then be included beside or within all pictures you take at each magnification to provide a reliable scale or even super-imposed on the picture as a scale bar. This can be accomplished with Photoshop or similar program. Once the scale is attached to the original image, the image size can be changed and the scale will change with it. Be sure to label the length of the scale bar on the picture.

An alternative to the Photoshop cut-and-paste approach, suggested by Andrew Spink (Bryonet 16 April 2010) is to use the free software **Combine ZP** <http://www.hadleyweb.pwp.blueyonder.co.uk/CZP/Installation.htm>. If you know the actual distance between two points on the photo, this program can add a scale bar (Figure 38). This same program is designed to stack several photos taken at slightly different focus, but it also permits this ability to stack a scale bar.

Figure 38. ***Lophocolea heterophylla*** with scale bar added using Combine ZP. Photo by Andrew Spink.

Inserting Scales into Images Using Photoshop

David Wagner provides us with a means of making a set of scales to use for various magnifications.

In Photoshop, open three files:

1. page with scales (Figure 39)
2. subject
3. image of stage micrometer at same magnification as subject. (Wagner photographs a stage micrometer at every photo session, at all the magnifications used in that session.)

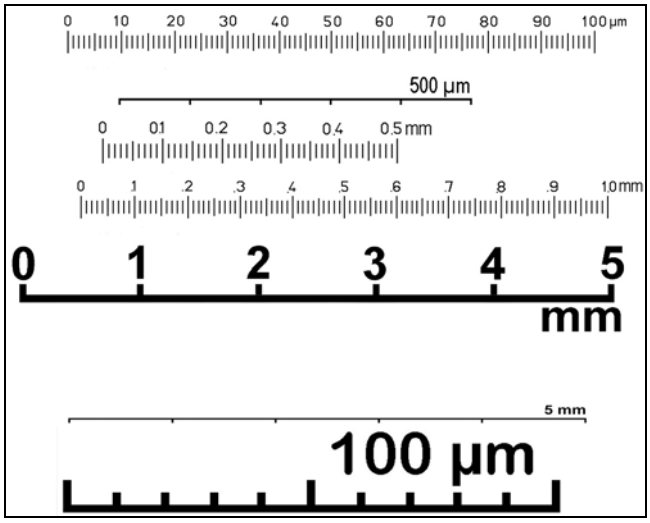

Figure 39. Scalebars that can used to label microscope images. Provided by David H. Wagner.

4. Bring file #1 (Figure 39) to the top and select the appropriate scale. (The top one, 100 µm, Wagner uses only with the highest magnification (40X objective), middle ones for 10X objective, and bottom ones for 4X and multi-image mosaics.)
5. Copy the selected scale to the clipboard.
6. Bring file #3 to the top and paste the copied scale onto the micrometer image. (see Picture 1, attached).
7. Use the move tool (in Photoshop CS3 you need also to set the move tool with Edit/Transform/Scale) to adjust the scale to the micrometer.
8. Flatten layers (Layer menu).
9. Select the calibrated scale and paste it onto the subject image (Figure 40). NEVER resize images before the calibrated scale is pasted in!

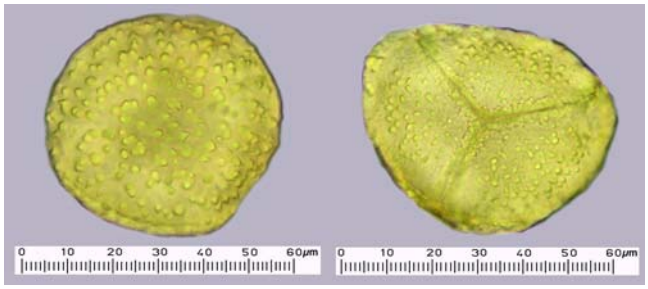

Figure 40. ***Phaeoceros oreganus*** spores with image stacking and added scalebar (from Photoshop) using both transmitted and reflected light. Photo by David H. Wagner.

Stacking

Manual stacking, using Photoshop, is also possible, albeit more time-consuming. Stacking in photography is the process of taking multiple images of the same subject, each at a slightly different focus. Software such as Combine ZP is used to put the images together, using the best focus portions of each to make a combined image with outstanding depth of field (Figure 41-Figure 42). This approach is also known as **deep focus** (David H. Wagner, Bryonet 19 April 2010).

Figure 41. Gemma cup of ***Marchantia polymorpha*** showing all gemmae in focus, a result of stacking 8 images in Photoshop. Note that the 1 mm text is hidden by the dark background. Text on a dark background should be in white or black framed in a white box. Photo by David H. Wagner.

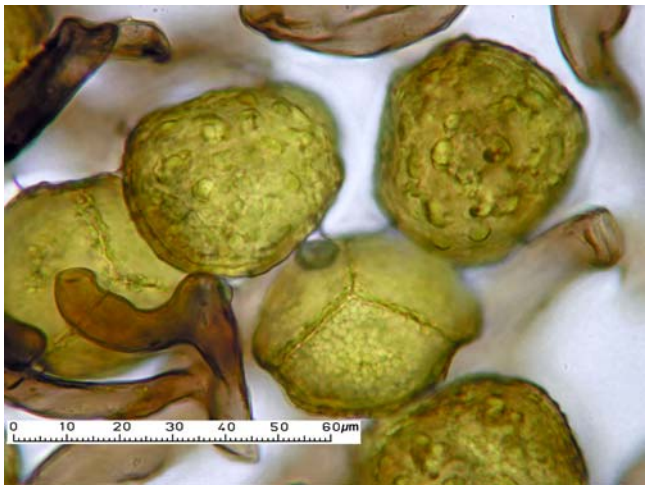

Figure 42. ***Phaeoceros pearsonii*** spores with image stacking and scalebar, using both transmitted and reflected light. Photo by David H. Wagner.

In creating the images in Figure 41-Figure 42, David H. Wagner (Bryonet 16 April 2010) used this stacking technique to create greater focus for the image of ***Marchantia polymorpha*** gemmae (Figure 41). This image was created from a stack of eight images, adding one image at a time. The spore image in Figure 42 used stacking with both transmitted and reflected light to make the details clearer.

Norbert Stapper demonstrates the use of stacking to photograph leaves and other parts of a moss under the microscope (Figure 43-Figure 44). He likewise used Combine Z.

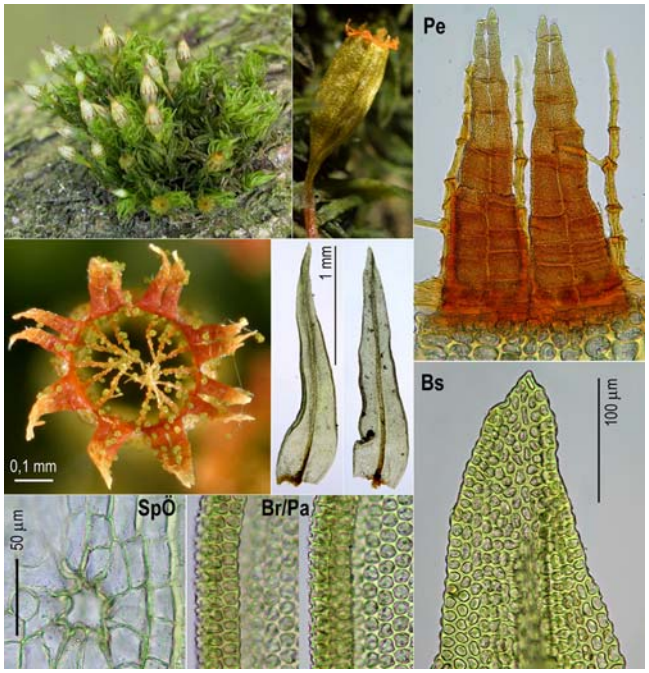

Figure 43. Stacked images, using Combine Z, of ***Orthotrichum patens***. Photos by Norbert Stapper, with permission.

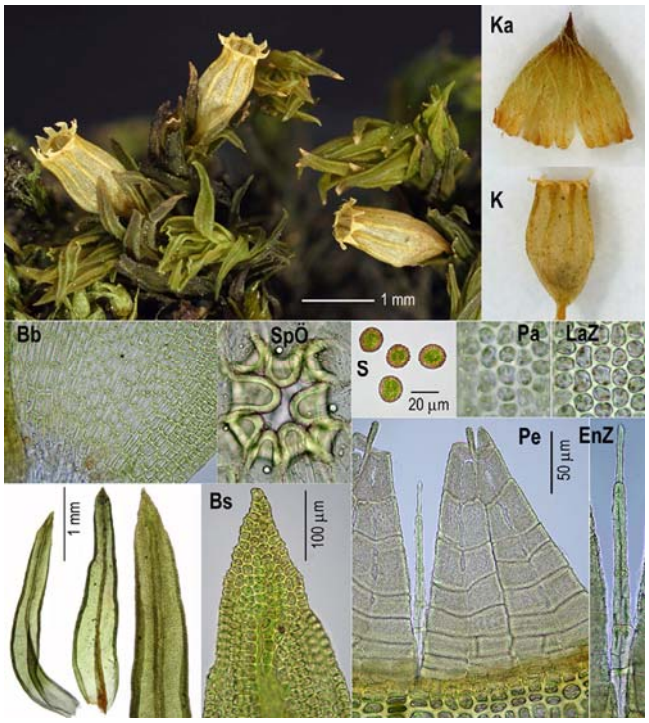

Figure 44. Stacked images, using Combine Z, of ***Orthotrichum pulchellum***. Photos by Norbert Stapper.

Standardizing Focus Increments For Image Stacking Photomicrography

Stacking software has reached widespread use in bryology because it makes it possible to greatly increase the depth of field for these small objects. Its principle is to take a series of images, each at a slightly greater focal distance. These are combined using the best focus areas of each with software such as Z or built-in software in the Olympus Tough camera. Objects such as bryophyte shoots photographed in reflected light with the clear focus of stacking have a three-dimensional appearance that can be very beautiful. This set of instructions is only slightly modified from those of Wagner on Bryonet (19 April 2010).

The stacking software works best if photos are taken in evenly graduated, overlapping focal planes. When the overlap is optimal, usually about 25%, neither too many nor too few pictures need be taken. Precise, expensive equipment is available that performs this process automatically. But obtaining excellent results by careful manual focusing is easily managed. Wagner has installed a handmade metering dial on his microscope that has served well in this regard.

Draw a **circle** on a card and divide it into **10°** segments using a protractor (Figure 45). Draw in the **radii** for each segment (Figure 46). There is nothing special about 10°; it is simply convenient and easy to see for this process.

Cut out the center of this dial, using a hole diameter that will fit around the fine focus knob on one side of the microscope (Figure 46).

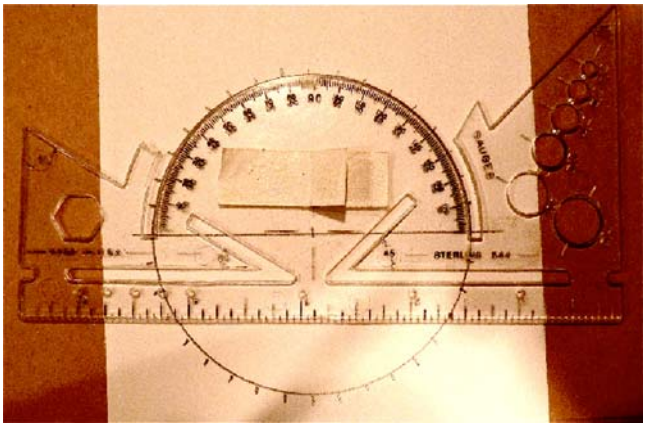

Figure 45. Protractor Photo by David H. Wagner.

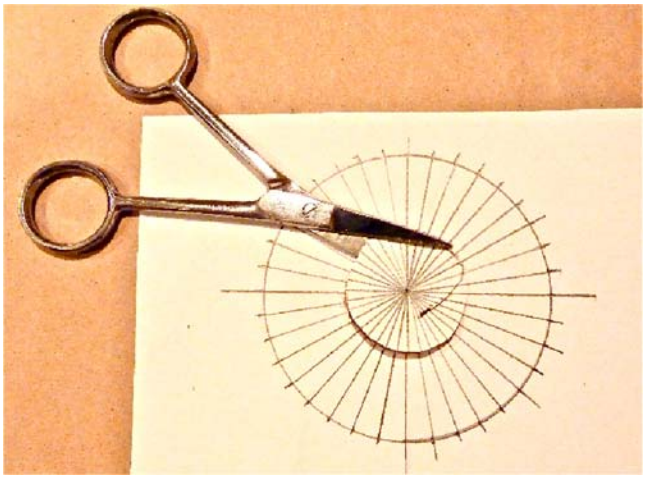

Figure 46. Card with 10° increments of radii. Photo by David H. Wagner.

Trim the card to fit and tape it to the microscope so that the fine focus knob is centered in the middle of the card's dial (Figure 47). Attach a "needle" firmly to the center of the focusing knob, so that the needle reaches to the edge of the dial. Wagner has used artist's putty to

attach it and for a needle has used a piece of black binding tie that comes with power cords. Plastiline modeling clay and a toothpick or bobby pin would work about as well.

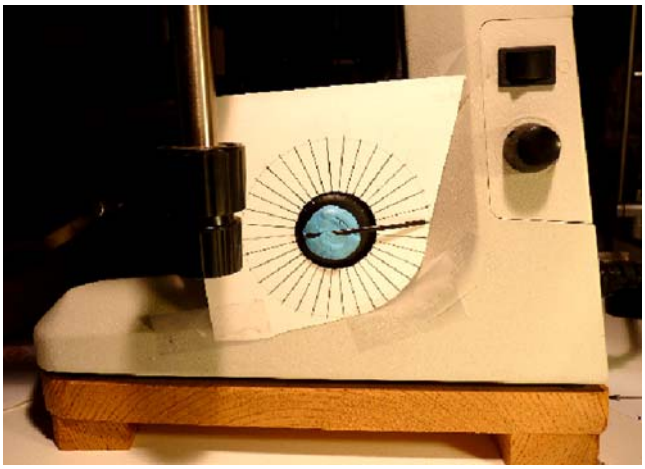

Figure 47. Stacking radii taped to microscope. Photo by David H. Wagner.

Mount a mirror in such a way that the needle and dial are visible as you use the microscope (Figure 48). Although this is not essential, it helps to be able to monitor the view through the microscope, adjusting focus with one hand while taking photographs with the camera's remote shutter release in the other hand.

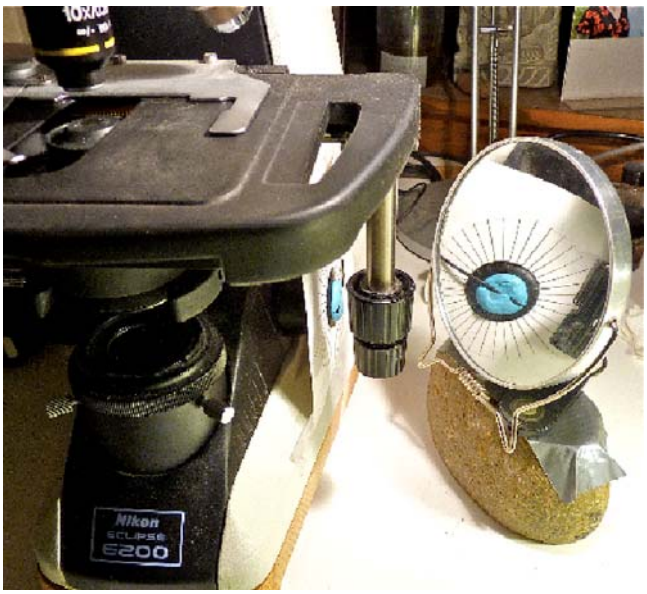

Figure 48. Mirror mounted so that needle and dial are visible while using the microscope. Photo by David H. Wagner.

For any particular object, first determine how many turns around the dial are necessary for a complete series of images. Focus on the top of the object and note where the needle is on the dial. Then focus down to the lowest focal plane you want to capture to determine the number of revolutions needed. For most slide-mounted objects it usually requires 1-3 complete revolutions around the dial to focus from top to bottom (near to far) focal planes.

Experimentation is necessary to use this system effectively. Wagner has found that with a Nikon Eclipse E200 microscope and a Nikon Coolpix camera, focus levels that work well are:

4X objective: 6 increments of the dial per image
10X objective: 2 increments of the dial per image
40X objective: 0.5 increments, or even better is to take three images per increment. (Oil-bodies in liverwort cells come into and out of focus with very slight touches of the fine focus knob.)

Practice is needed for consistent results. These guidelines provide about 25% image overlap, which works well with Helicon Focus. Maximum resolution settings on the camera help the stacking program to work optimally.

Once the object is properly staged, keep an eye on the dial and an ear on the shutter sounds. Turn the knob for the pre-determined number of increments with one hand on the fine focus knob, then activate the shutter release with other hand, turn the focus to the next stop, push the shutter release, *etc.*, until you have completed the number of revolutions of the needle on the dial to make a complete set of images from top focus to bottom focus.

For most bryophyte images, 10-30 images are likely to be adequate. Nothing is lost by taking extra images, and some are likely to be discarded. The useful ones are exported to a stacking program such as Combine Z. Once the stacking is completed, you can use Photoshop or other image management software to clean up debris, resize, sharpen, or whatever is needed.

Culture and Viewing Chamber

Paul Davison (2006; pers. comm. 22 February 2012; Davison & Kittle 2004) has made a viewing/photography cell that is useful for viewing aquatic bryophytes and bryophyte inhabitants (Figure 49-Figure 51). This uses two microscope slides with a spacer (foam) between them, temporarily bound together by vice or binder clips. The spacer must leave enough marginal space to squirt silicone between the panes as a sealant. Once the silicone sets, remove the spacer.

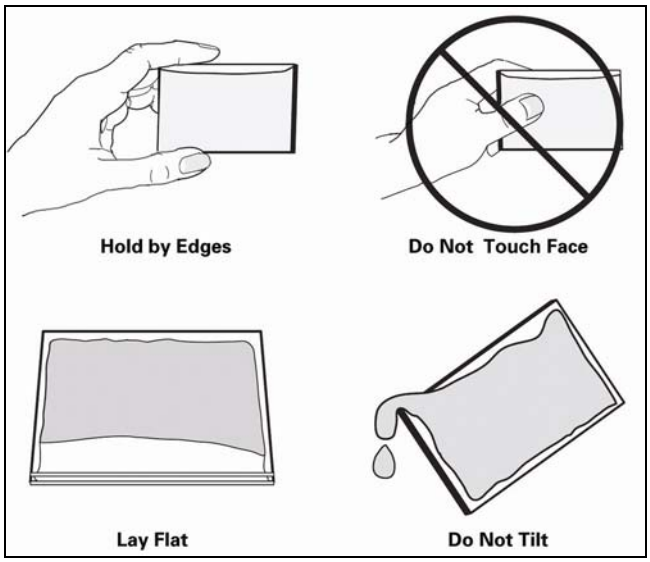

Figure 49. Method for constructing a microchamber for observing bryophytes and small invertebrates. This chamber can be used for projecting the images on a screen for teaching purposes. Modified from Davison 2006.

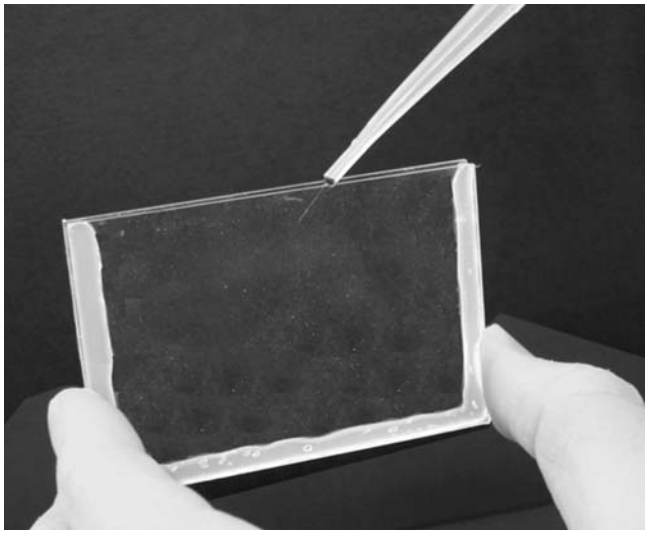

Figure 50. Filling completed microchamber built by above construction. Photo by Paul G. Davison from Davison 2006.

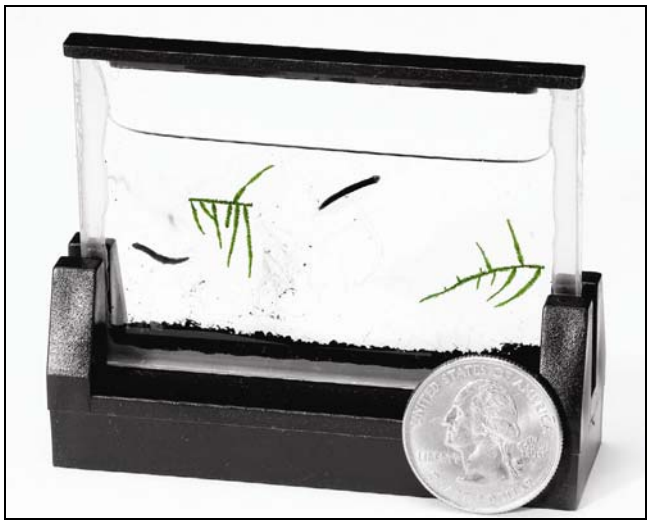

Figure 51. Occupied microchamber (with invertebrates and moss). Image modified from Davison 2006.

Summary

Adjust your microscope for the best possible viewing by adjusting the ocular distance and parfocus. Keep frequently used equipment at the microscope desk in a place that is easy to reach. Protect equipment such as microforceps against damage and sharpen tools as needed. Use a scalebar with the microscope and be sure that images have a scale reference.

Put small inhabitants into small chambers to minimize movement for photography. Use stacking equipment and software for the best images.

Acknowledgments

Bryonetters have been invaluable in providing the information used in this chapter.

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