CHAPTER 2-1
LABORATORY TECHNIQUES: EQUIPMENT

Janice M. Glime and David H. Wagner

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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.

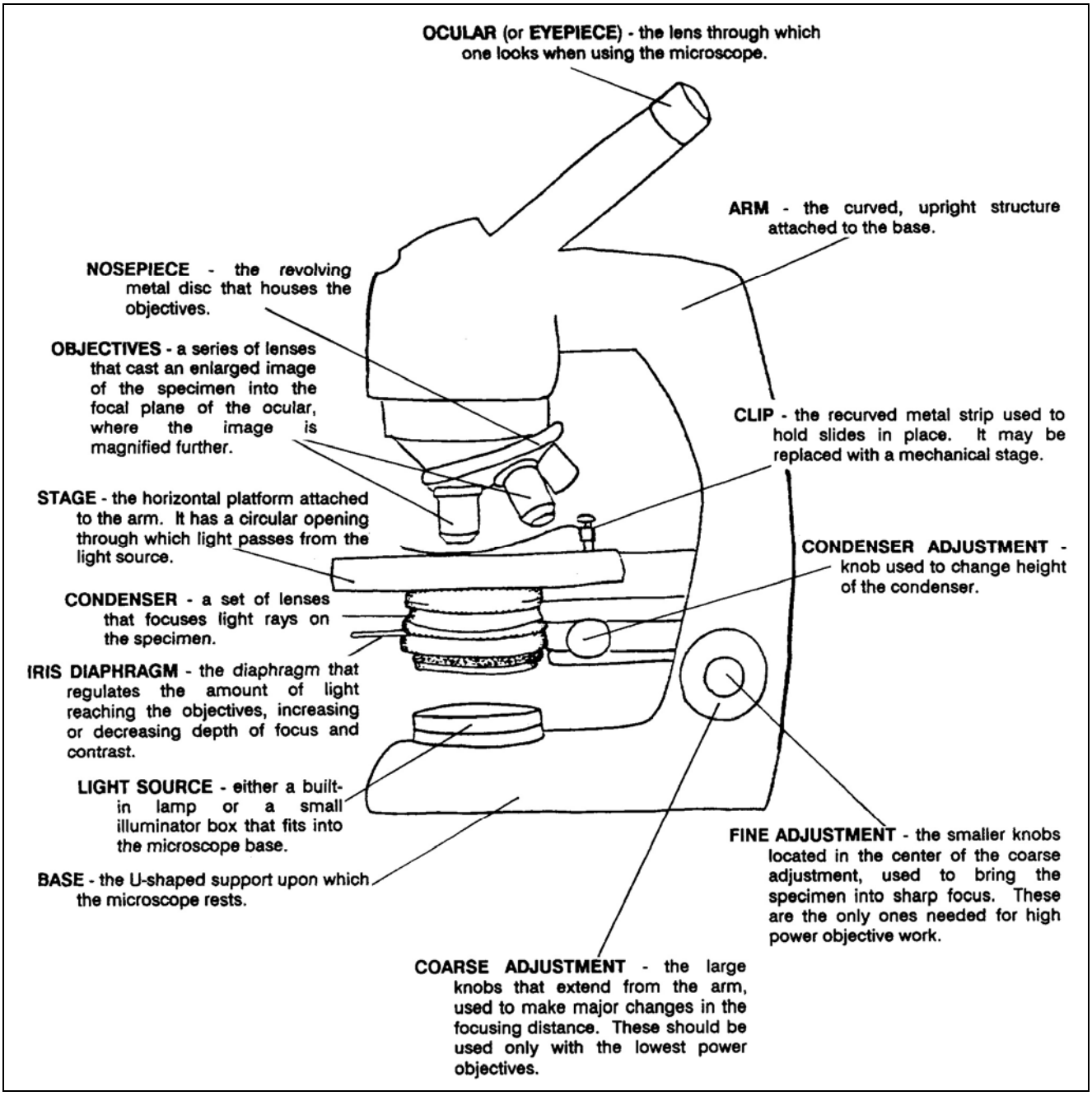

Figure 4. Compound microscope showing parts. Drawing by Janice Glime.

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.

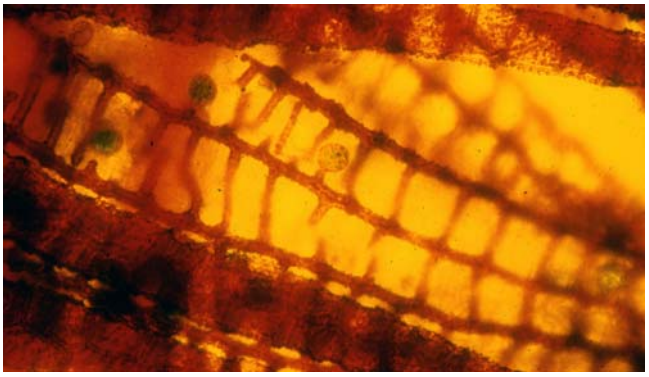

Figure 6. Peristome of ***Fontinalis squamosa*** with tungsten light of microscope and no blue filter. Photo by Janice Glime.

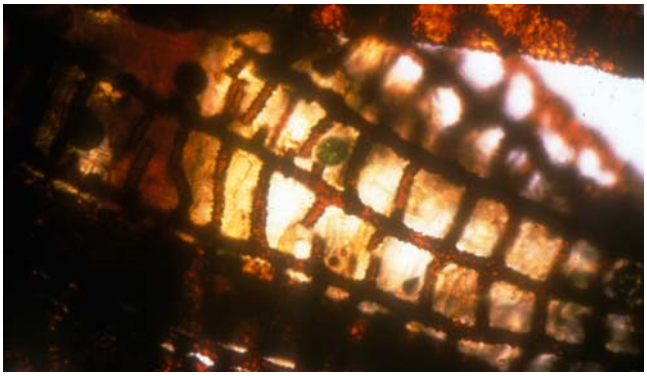

Figure 7. Peristome of ***Fontinalis squamosa*** with blue filter covering tungsten light of microscope. Photo by Janice Glime.

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.

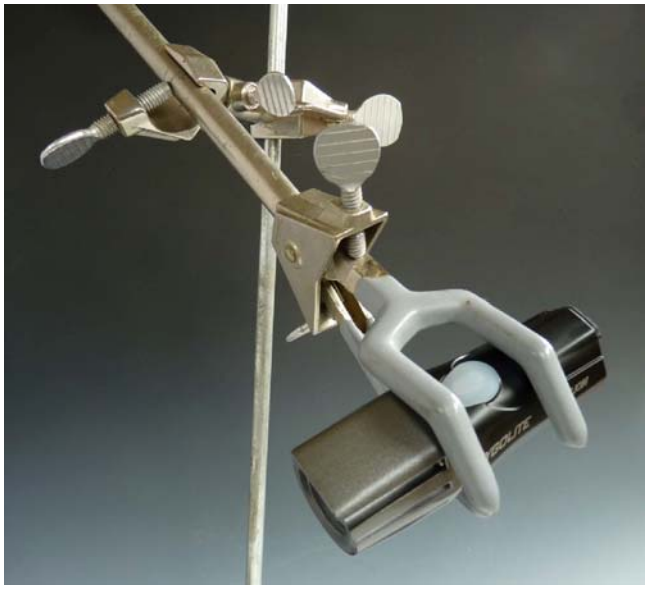

Figure 12. LED light source mounted for use with a microscope. Photo by David H. Wagner.

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).

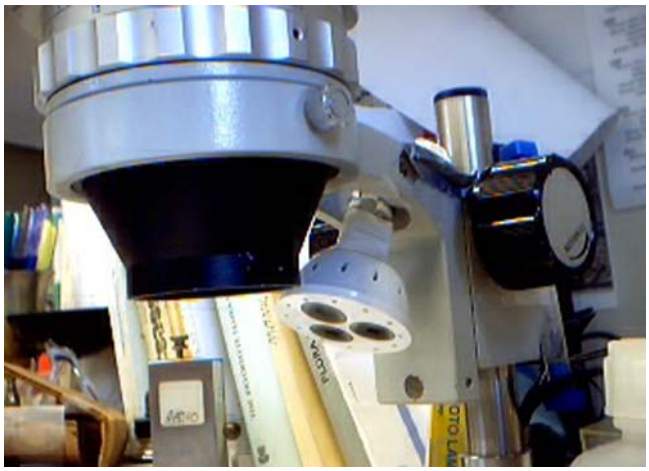

Figure 13. Triple 1-watt Luxeonwarm-white LED unit mounted on dissecting microscope with duck tape. Photo by Richard Zander.

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.

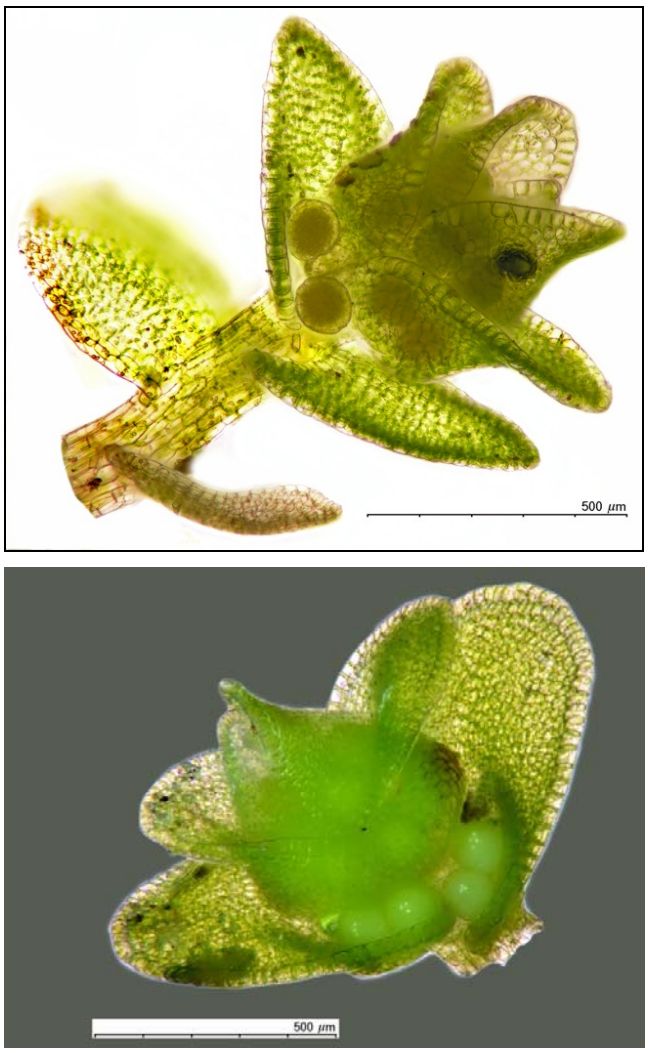

Figure 14. Comparison of light sources for microscopy. Note pasted-in scalebars. **Upper:** transmitted light. **Lower:** transmitted + reflected light. Photos by David H. Wagner.

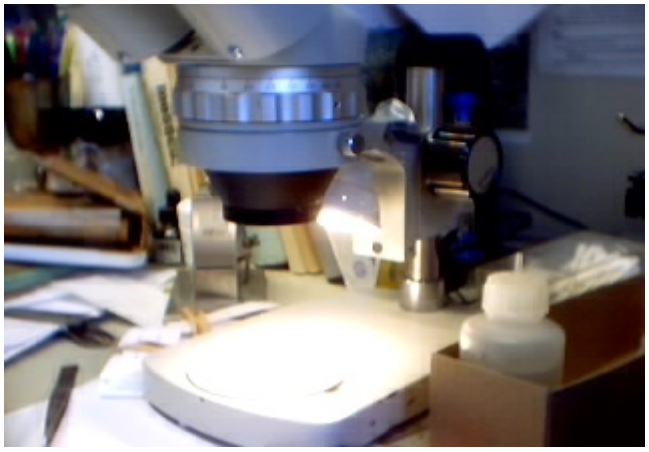

Figure 15. This unit provides an even, warm white light spread over the base of the microscope. Photo by Richard Zander.

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**.

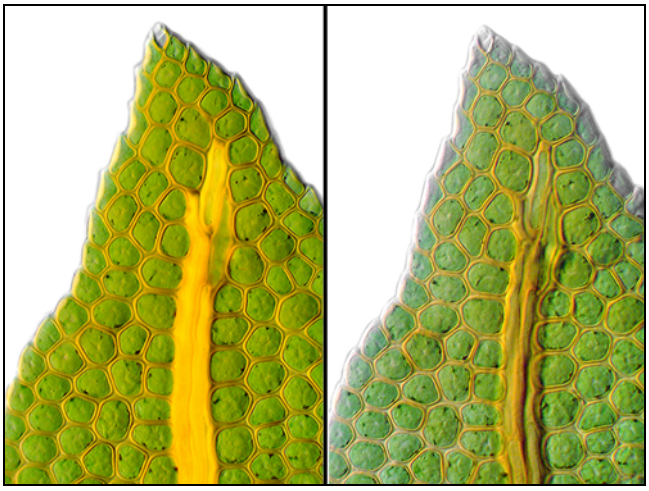

Figure 17. ***Fissidens pusillus*** showing differences in detail in light microscopy (**left**) and **ha'penny microscopy** (**right**). Photos by Bill and Nancy Malcolm.

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).

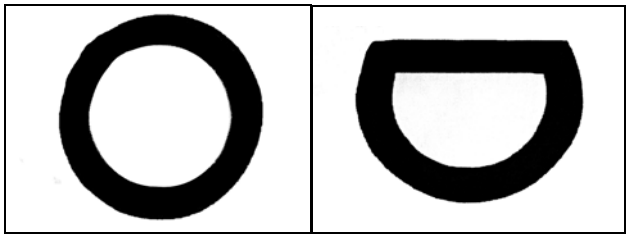

Figure 18. Ha'penny differential interference paper of Malcolm and Malcolm. **Left:** O-shaped interference for flat lenses. **Right:** D-shaped interference that may work better on curved lenses. Drawing by Janice Glime.

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.

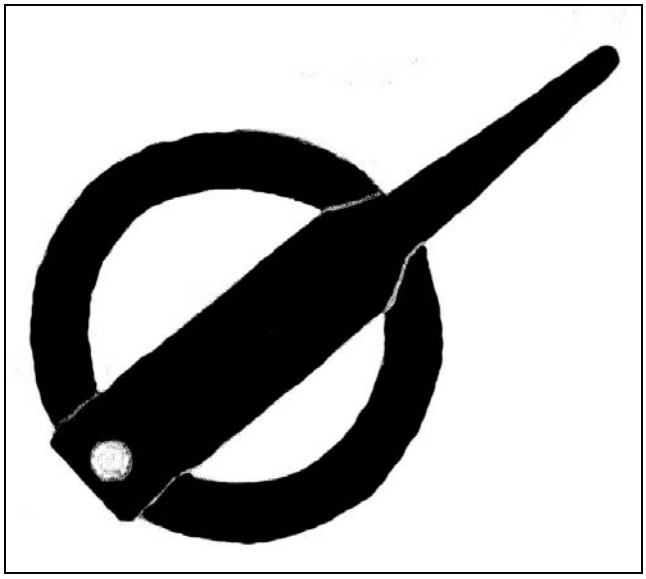

Figure 19. Ha'penny differential interference paper of Malcolm and Malcolm with lever attached. Drawing by Janice Glime.

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.

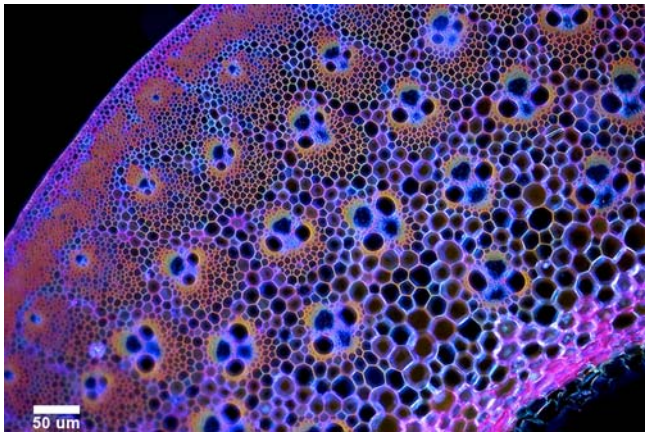

Figure 20. *Fargesia* sp. (bamboo) stem cross section in polarized light. Photo by Eckhard Völcker through Flickr.

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.

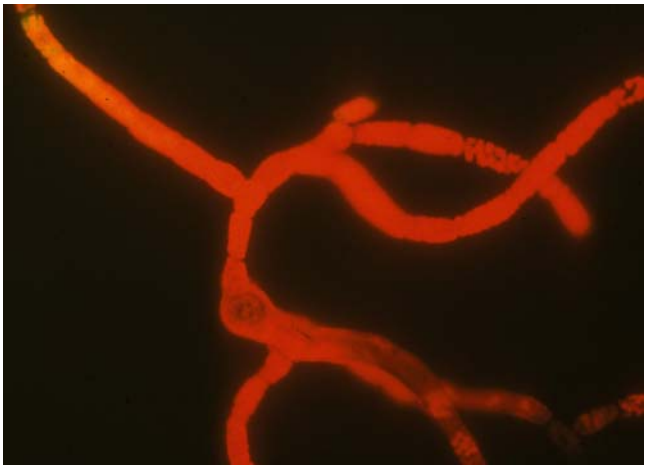

Figure 22. ***Fontinalis squamosa*** protonema using fluorescence microscopy and UV light source. Note that the chlorophyll fluoresces red. Photo by Janice Glime.

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.

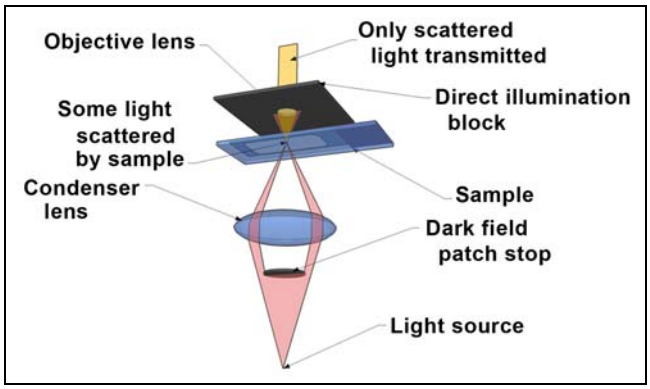

Figure 23. Dark field microscopy setup. Illumination modified from Wikimedia Creative Commons.

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).

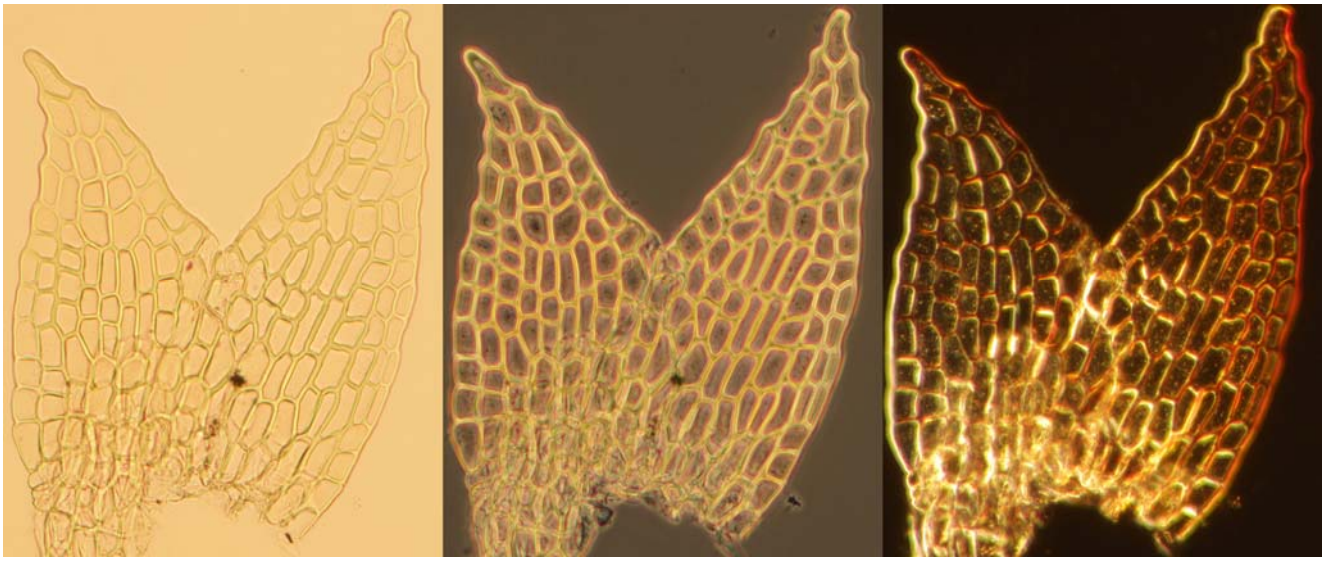

Figure 24. Comparison of microscopical techniques of ***Anthelia juratzkana***. **Left:** light microscopy. **Middle:** phase contrast microscopy. **Right:** dark field microscopy.

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.

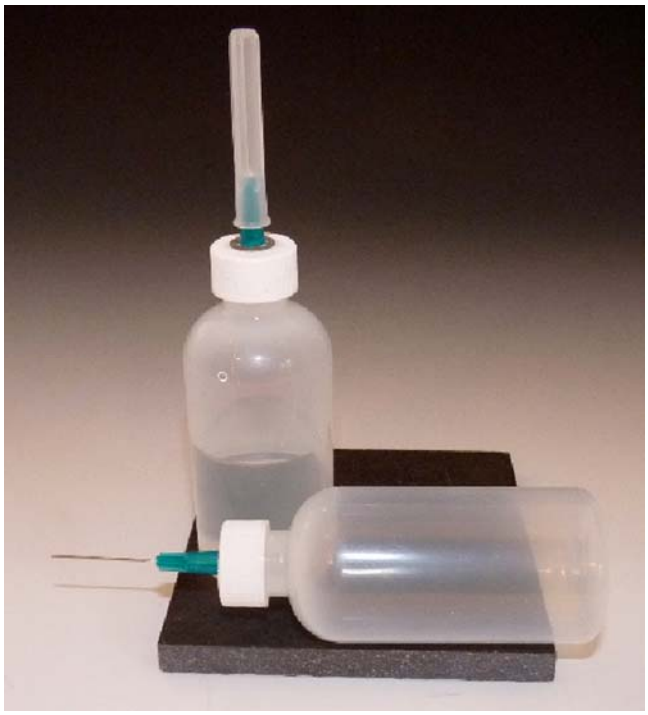

Figure 29. Needle dropper bottle for wetting mosses or adding water to a slide. Photo by David H. Wagner.

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).

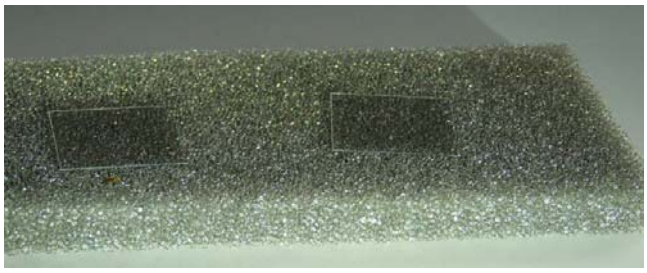

Figure 30. Polyurethane foam for holding microscope slides. Photo by David H. Wagner.

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.

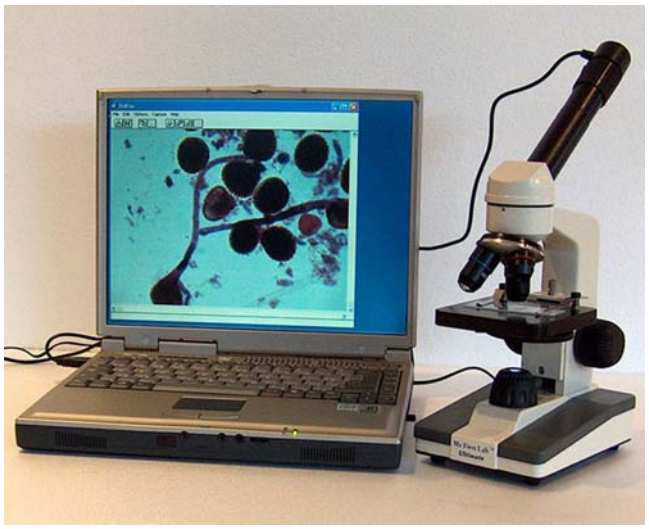

Figure 36. Computer screen with USB hookup to microscope. Photo by GeckOOptical. PERMISSION PENDING

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.)

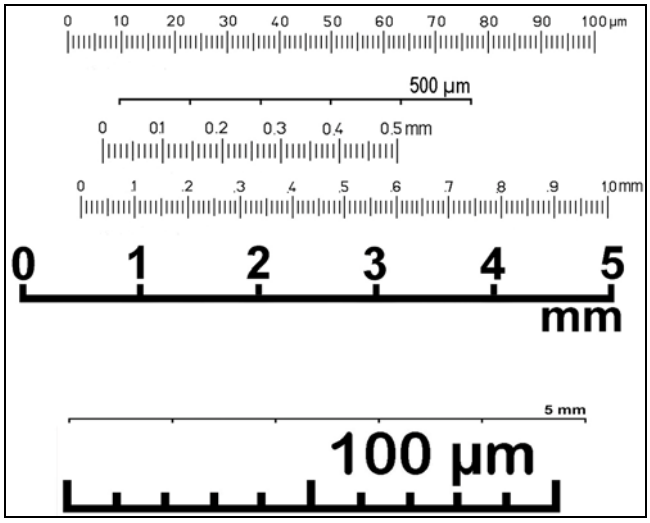

Figure 39. Scalebars that can used to label microscope images. Provided by David H. Wagner.

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!

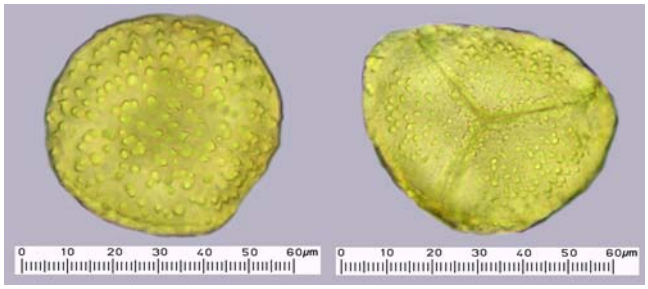

Figure 40. ***Phaeoceros oreganus*** spores with image stacking and added scalebar (from Photoshop) using both transmitted and reflected light. Photo by David H. Wagner.

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.

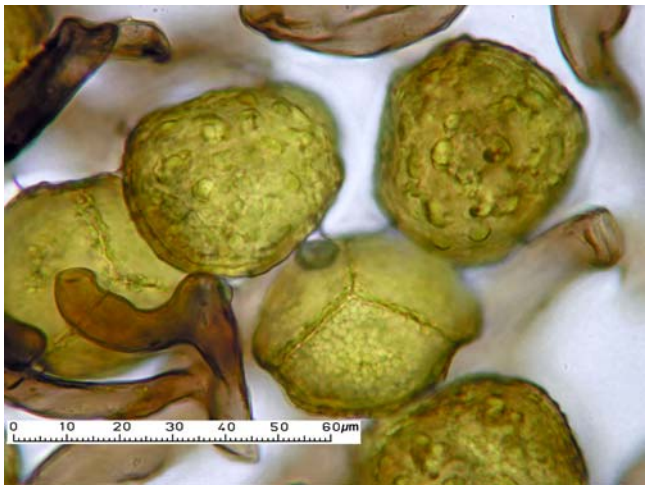

Figure 42. ***Phaeoceros pearsonii*** spores with image stacking and scalebar, using both transmitted and reflected light. Photo by David H. Wagner.

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.

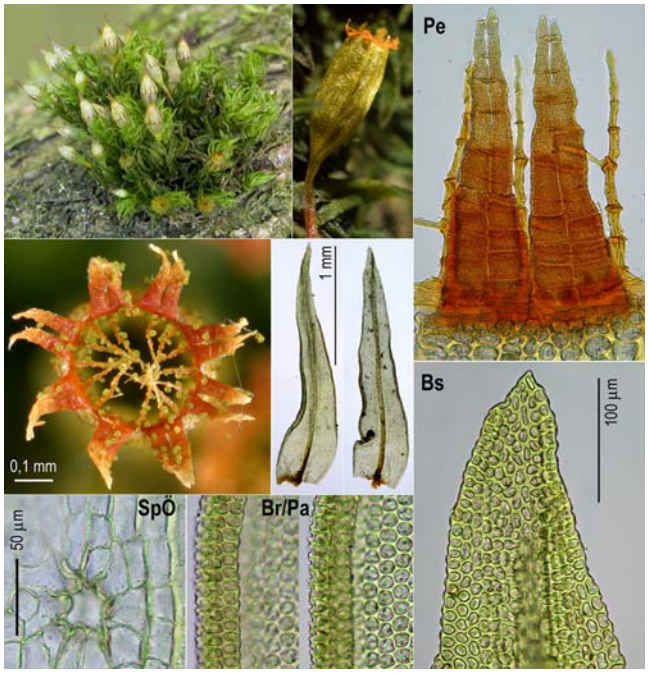

Figure 43. Stacked images, using Combine Z, of ***Orthotrichum patens***. Photos by Norbert Stapper, with permission.

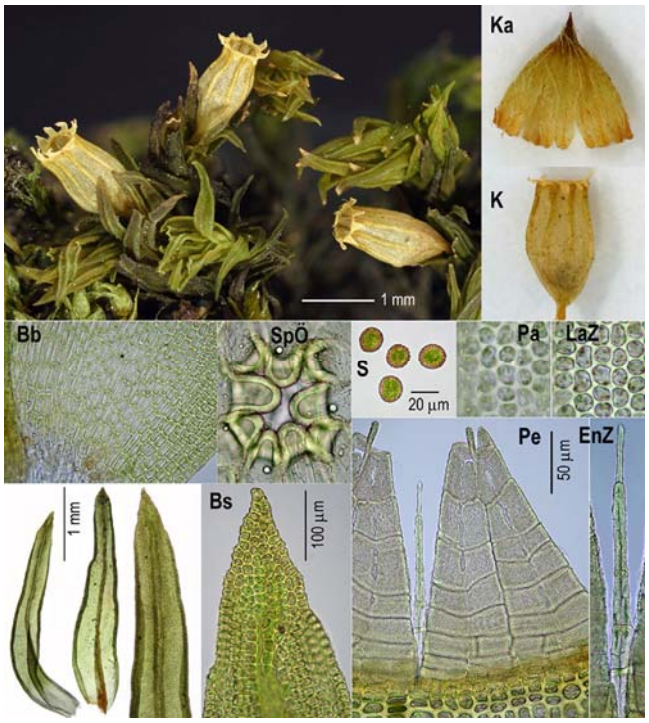

Figure 44. Stacked images, using Combine Z, of ***Orthotrichum pulchellum***. Photos by Norbert Stapper.

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).

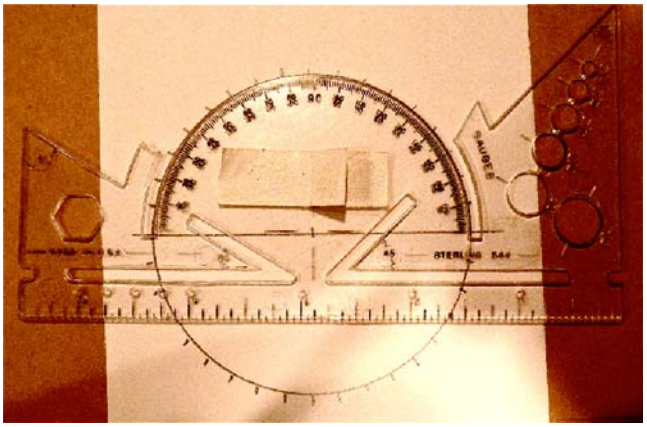

Figure 45. Protractor Photo by David H. Wagner.

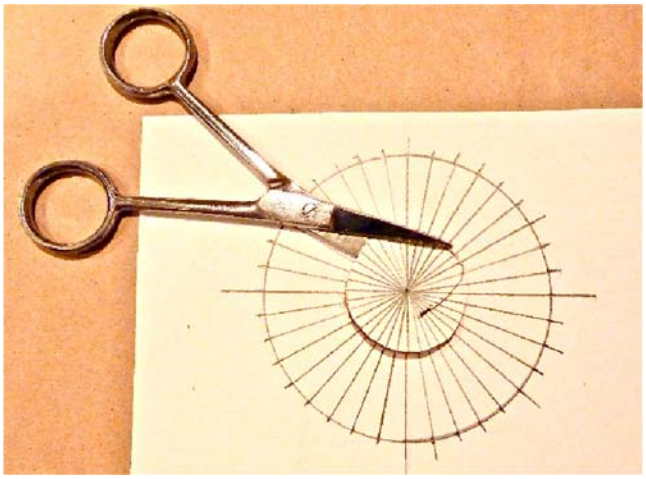

Figure 46. Card with 10° increments of radii. Photo by David H. Wagner.

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.

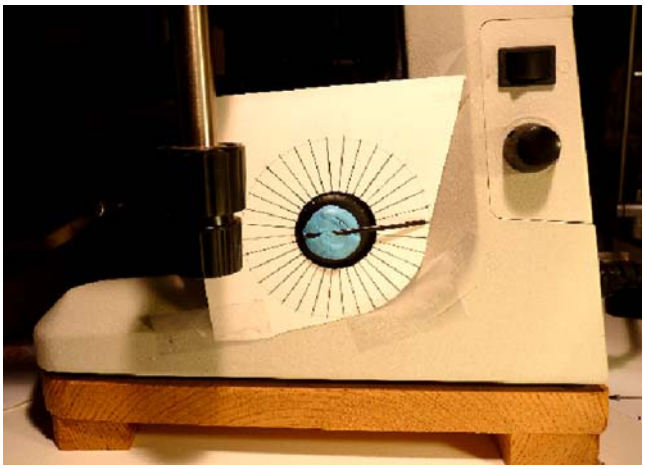

Figure 47. Stacking radii taped to microscope. Photo by David H. Wagner.

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.

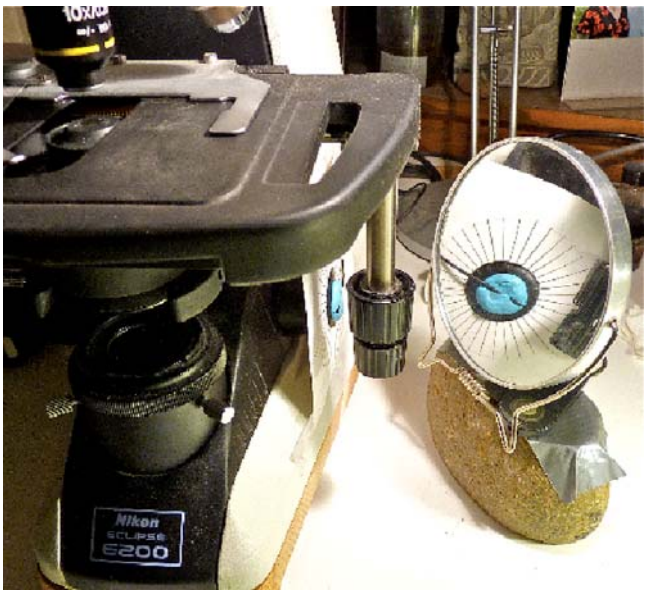

Figure 48. Mirror mounted so that needle and dial are visible while using the microscope. Photo by David H. Wagner.

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.

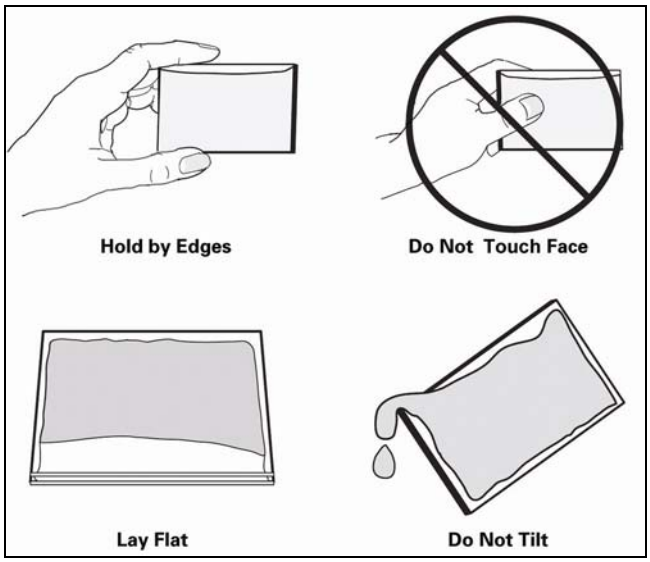

Figure 49. Method for constructing a microchamber for observing bryophytes and small invertebrates. This chamber can be used for projecting the images on a screen for teaching purposes. Modified from Davison 2006.

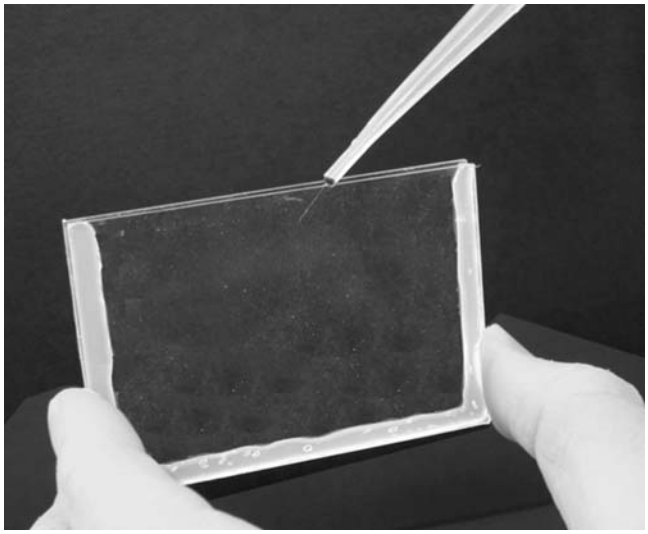

Figure 50. Filling completed microchamber built by above construction. Photo by Paul G. Davison from Davison 2006.

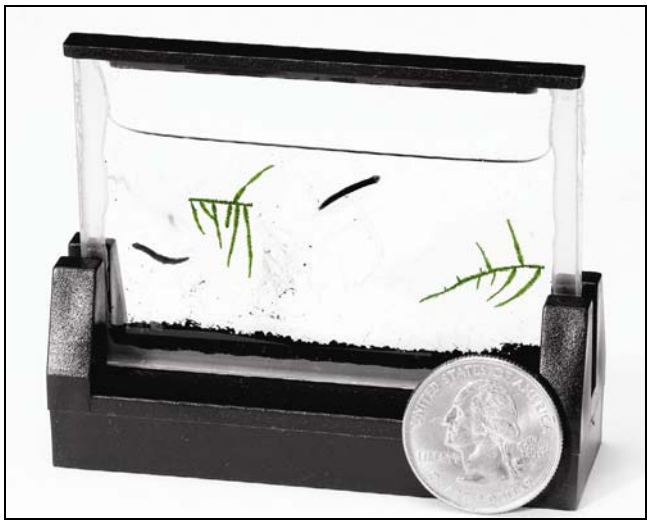

Figure 51. Occupied microchamber (with invertebrates and moss). Image modified from Davison 2006.

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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CHAPTER 2-2a
LABORATORY TECHNIQUES: SLIDE PREPARATION AND STAINS

Janice M. Glime and David H. Wagner

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CHAPTER 2-2a
LAB TECHNIQUES:
SLIDE PREPARATION AND STAINS

Figure 1. ***Polytrichum juniperinum*** leaf cross section using a cryostat and displaying natural colors. Photo courtesy of John Hribljan.

Preparing the Specimen

Fresh specimens are the most fun to work with. They are bright green and require little or no hydration before placing them in a drop of water on a slide. Chloroplasts migrate in cyclosis. And tiny invertebrates crawl about to entertain and distract you. But most often we don't have the pleasure to observe fresh material under the microscope. Instead, we have dry, often brittle, specimens collected in great numbers in a day-long or even months-long collecting trip. But don't dismay – the bryophytes will still freshen up to make good slides.

Cleaning Bryophytes

Washing Machine (Jewett 1913)

Jewett (1913) suggests a small "**washing machine.**" The bryophytes are placed on a fine screen – we assume that cloth window screening would work – and sprayed with a nozzle to clean them.

Embroidery Hoop (Mayfield *et al*. 1983)

Mayfield *et al*. (1983) suggested a similar cleaning procedure using a net, but they suggested placing the netting (mosquito or bridal veil netting) tightly in an **embroidery hoop**. This is particularly useful for thallose liverworts. They should be collected with ~3 mm substrate to protect rhizoids and scales. The liverworts are placed on the hoop netting with a second net placed over them. They are then washed with a stream of water. This may take some practice because too much water will damage the plants whereas a weak stream will not succeed in removing the soil and debris. Mayfield and coworkers suggest that a suitable stream of water can be achieved by attaching an eyedropper to pliable tubing. If the tubing is connected to a tapered laboratory water faucet, water flow can be adequately controlled. Specimens can then be pressed suitably in a telephone book, using folded waxed paper to hold the specimens between the pages of the book. Dried specimens are affixed to a 2x5" (5x12.5 cm) card with water-soluble glue. Specimens can be rehydrated when needed with boiling water. Contemporary workers discourage pressing or gluing specimens.

Wash Bottle (Wagner 2011)

Wagner (2011) suggests having a small **wash bottle** (125 ml) for rinsing the bryophytes and cleaning slides and coverslips for reuse (Figure 2). The water can also be used to wash away the wetting agent. The same ability of a wetting agent (see below) to reduce trapped bubbles also causes the water drop on your slide to lose its cohesion and adhesion, causing the water drop to run all over the place, so start with a small drop.

Figure 2. Water bottle and ceramic washing cup. David Wagner says he likes "to use a pretty, wood-fired stoneware cup, much more pleasing to the eye than the usual beaker." Photo by David Wagner.

HCl (Zander 1993)

Zander (1993) suggests using dilute HCl to clean away limy incrustations. It can also indicate whether the collection was made from a calcareous habitat because, if it is calcareous and bits are present with the sample, it will produce bubbles.

Ultrasound

Jan-Peter Frahm (Bryonet 11 December 2013) suggested using a sonicator to clean bryophytes before making slides, especially when they are used for photography. The bryophyte can be suspended by forceps into the vibrating bath. These are available for cleaning jewelry and watches in small sizes at relatively inexpensive prices. I tried this for cleaning ***Fontinalis***, but it disrupted the cell contents without dislodging adhering diatoms like ***Cocconeis*** (Figure 3-Figure 4). On the other hand, Randal Mindell (pers. comm. 2 January 2022) successfully cleaned ~24,000-year-old bryophyte subfossils.

It might be helpful to add a cleaning agent to the water to facilitate removal. Rod Seppelt (Bryonet 11 December 2013) suggests Tween 80 as a detergent. He dilutes it (lots) with water to clean capillary ink pens.

Aquatic Bryophytes (Landry 1973)

Aquatic bryophytes can be particularly challenging. They typically are covered with epiphytes, some of which (*e.g.*, the diatom ***Cocconeis***; Figure 3-Figure 4) embed themselves into the surfaces of the leaves. Landry (1973) experimented with various cleaning techniques on ***Fontinalis*** (Figure 5). He found that household bleach, diluted to 0.5%, causes no chlorophyll bleaching, but at 0.10% bleaching appeared in 15 seconds. Solutions diluted to 0.25% caused bleaching in 5 seconds. Bleaching in these cases occurred in the lower (older) leaves and may have been tied to senescence. Unfortunately, these methods did not appear to remove the epiphytes. Ultimately, 5 minutes cleaning with 3% peroxide (H_2O_2) and agitation seemed to remove approximately 85-90% of the epiphytes (Figure 5). Tumbling the moss at 30 revolutions per minute still only removed only about 85% of the epiphytes. Swirling improved the removal. Higher concentrations of peroxide and/or longer time periods caused bleaching of the chlorophyll.

Figure 3. ***Cocconeis placentula*** cemented to an algal strand. Photo by Bernd Kaufmann, through Creative Commons.

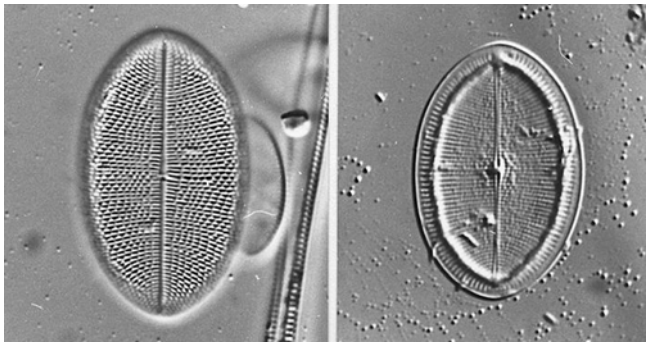


Figure 4. Both valves of ***Cocconeis placentula***, a common diatom that imbeds itself into the cell wall of aquatic bryophytes. Photo by Pauli Snoeijs, Nordic Microalgae <www.nordicmicroalgae.org>, through Creative Commons.

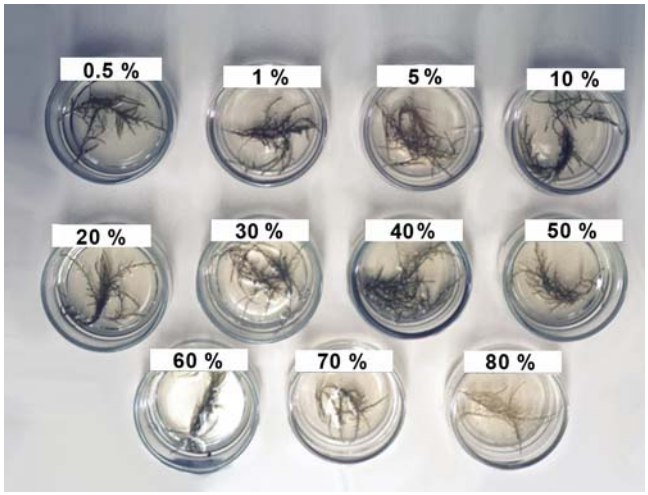


Figure 5. Experiment on cleaning ***Fontinalis*** sp. with household hydrogen peroxide at various concentrations. Note the bleaching at 70 and 80%. Photo by Janice Glime.

Dealing with Old Specimens

Old samples can be brittle and fragile. Placing them in water to soak can further degrade them so that they fall apart when cut. Adam Hoelzer (Bryonet 11 January 2016) takes from an old sample a single branch and puts it on a slide in a drop of water without soaking. Even if the branch is flattened from storage in a packet, that is helpful. Under a stereo microscope, Hoelzer keeps the branch affixed with his left index finger in position in the drop of water and cuts thin slices of the branch with a single-edged razor blade. You need some experience but that is the quickest and easiest way. Do not soak the parts as they get very soft. There is no need of using *Sambucus* or anything else to hold the moss. After cutting you can heat the sections carefully for swelling for a very short time with the help of a very small amount of KOH under the cover slip.

Sorting the Plants

A classic mistake in identifying bryophytes is looking at the sporophyte of one species and the leaves of another. Sporophytes often originate deep in the clump and may actually belong to a species that achieved sufficient dominance in a previous year to produce a capsule. But another species can easily encroach or simply intermix enough to confuse the unwary. Be sure to track the sporophyte down and locate its attached gametophyte. You might find it belongs to a small pleurocarpous moss that is weaving in and out among your acrocarpous cushion. This sorting should be done with bryophytes that are moist enough to be soft, but not soaked. Dry mosses are likely to break before you can pull the gametophyte out from among its trappings.

Wetting Agents

Assuming your specimens have not been collected in the same day and have gotten dry and brittle, the first step is to re-wet them before attempting to make a slide or even examine them with the dissecting microscope. Dry bryophytes are often brittle and will break easily if you begin manipulating them without wetting them first. However you wet them, we recommend watching them with a dissecting microscope as the water moves through the capillary spaces among the stems. It is a fascinating display and is sure to grab the attention of first-time viewers such as students.

Most bryophytes will wet up adequately by dipping them in water or dropping water or misting on the desired portion of the sample. Once the specimen has regained its wet shape and is pliable, leaves can be removed by holding the tips of the stems with a pair of forceps (can be ordinary lab forceps if the specimen is not tiny) or a dissecting needle (probe) and a second pair of microforceps should be used to pull down on the desired leaf, being careful to hold the leaf in a position close to the stem to get as much of its base as possible. For smaller species, curved microforceps often work best for holding the stems.

But some mosses simply don't wet well. In fact, some bryophytes repel water and may even trap large air bubbles that further keep them from getting wet. Members of the **Mniaceae** (Figure 6) are notorious for this, and **Polytrichaceae** (Figure 7) can be contrary as well if water cannot reach the leaf bases easily. In particular, wetting agents help to avoid the air bubbles trapped in leaf folds by reducing or eliminating the surface tension of the water. Warm water can sometimes actually increase the bubbles. Soap is a wetting agent, and it doesn't take much. One drop in your dropper bottle is likely to be more than needed. But beware, soap and the other wetting agents, as well as heating, will usually kill the bryophytes and destroy the cell contents.

Figure 6. ***Plagiomnium*** sp. branch resisting wetting. Photo possibly courtesy of Derek Bewley.

Figure 7. ***Pogonatum urnigerum*** (**Polytrichaceae**), a genus where water must enter the leaf bases where there is less wax. Photo by David Holyoak, with permission.

One solution to getting some of these to get wet is to drop them in **hot (not boiling) water** (Jewett 1913; Lucas 2009). I have to wonder if this distorts anything, and it most likely melts waxes such as one might find on the Polytrichaceae. But it does make most of them flexible rather quickly, and lost wax is usually not a problem. Some bryologists actually keep a hot plate nearby with hot water while they work.

Koponen (1974) dips members of **Mniaceae** (Figure 6) into 70-90% ethanol, then into 2% KOH, ultimately washing away the KOH with water. The specimens are ready for examination in one minute and the chloroplasts are destroyed, making other cell contents visible and the cell walls a yellowish to brownish color. This is especially helpful when the corners of the cells must be seen clearly.

A traditional wetting agent is one known by the German word **Pohlstoffe**. This is a non-technical name for a wetting agent (di-octyl sodium sulfosuccinate) available from Fisher Scientific, known as **Aerosol OT** (Wagner 1981; Bryonet 23 July 2008); it is mixed in a 1:24:75 ratio with methanol and water. Wagner suggested omitting the methanol, finding that this modified mix brings leafy bryophytes, dry capsules, and peristomes to turgidity rapidly, virtually everything except thallose liverworts. Schofield (1985) likewise suggested using only Aerosol OT and water with a dilution of 1:100. It is named for Richard Pohl (Diana Horton, Bryonet 19 September 1999) who presented the formula as a softening agent for dried plant parts (Pohl 1954).

Wagner recommends a half dropper of the 10% solution in 50 ml of water in a dropping bottle. The Aerosol OT can be difficult to obtain, especially if you are not affiliated with an institution. A Google search only located sites that sold it in huge quantities at costs of $500 or more. Wagner (Bryonet 11 May 2010) learned from his students that the critical substance is also known as **docusate sodium**, the active ingredient of stool softener! Hence, it is available at the drugstore for about US $5.00 for 60 caplets (Figure 8). Wagner determined that one caplet with a liquid center (100 mg docusate sodium), not solid pills, in 25 ml of water works well as Pohlstoffe. The carriers (glycerine, gelatin, propylene glycol, polyethylene glycol) do not appear to leave any noticeable residue.

Figure 8. Examples of stool softeners with docusate sodium. Photos modified by Janice Glime.

Soap (Tom Thekathyil, Bryonet 12 May 2010)

Another solution to wetting bryophytes is to use **soap** or detergent as a wetting agent. Tom Thekathyil (Bryonet 12 May 2010) suggests diluted kitchen detergent. It doesn't take much. One drop in your dropper bottle is likely to be more than needed. **A word of caution**: Soap can destroy the oil bodies of leafy liverworts! Warm water with patience is a better approach, but hot water can destroy the oil bodies.

Agral 600 (Tom Thekathyil, Bryonet 12 May 1210)

Tom Thekathyil also uses **Agral 600** (horticultural wetting agent). The latter kills the animal life that often accompanies the bryophytes but does not seem to affect the plants. This is useful to avoid introducing dermestids and other hungry creatures into the herbarium.

Rehydrating Capsules

DulcoEase

Des Callaghan (Bryonet 28 January 2016) raised the question of rehydrating ***Weissia*** capsules (Figure 9) that are full of spores. He needed them to regain normal size so he could obtain measurements. He tried the laxative DulcoEase (containing docusate sodium) for rehydrating capsules, but in this case, the interior spores remained dry, presumably insulated from the water by the outer spores. This kept the capsule partially dehydrated and did not permit it to obtain its fresh size as needed for measurements.

Figure 9. ***Weissia controversa*** capsules. Photo by Michael Lüth, with permission.

Catherine La Farge and Stephen Rae (Bryonet January 2016) both suggested using hot water to rehydrate. David Wagner (Bryonet January 2016) suggested adding docusate to a 50% solution of ethyl alcohol. It can speed the rehydration. A closed capsule can be rehydrated more quickly by putting tiny pricks through the wall using an insect pin.

Terry McIntosh (Bryonet January 2016) suggested a variation on this technique. He puts a complete stem into a shallow glass or plastic dish and sprays them with a fine mist until the leaves open. Then he covers the dish with a tall, clear plastic or glass container and places it outside in a protected area. The next morning the plant appears field-fresh, including capsules.

A mild solution (5-10%) of dish soap (Dawn) in tap water, heated to ~90°C is good for hydrating liverwort capsules (and probably also works on moss capsules). The soap breaks up the surface tension (Scott Schuette, Bryonet January 2016).

Jessica Budke (Bryonet January 2016) had a more sophisticated suggestion. She puts capsules in a low-level vacuum while they are in solution. That is helpful in preparation for TEM. It also helps to cut the capsule lengthwise to excise more tissue. Arno van der Pluijm (Bryonet January 2016), when working with ***Orthotrichum***, first punctures the capsule with a minute insect needle into the spore sac. After adding water it then quickly rehydrates and nothing is damaged.

Howard Matcham and Jan Janssens (Bryonet January 2016) both use 2% KOH or stronger to soak capsules. This technique causes the lids to pop, but it is useful for viewing stomata. Matcham suggests a single drop onto a slide, then a 5 minute soak before viewing. Janssens suggests soaking in nearly boiling water before adding KOH.

Rather than KOH, Jurgen Nieuwkoop (Bryonet January 2016) uses a drop of alcohol in water to wet difficult tissues. Norbert Stapper (Bryonet January 2016) suggests using a humidifying air chamber at close to 100% relative humidity. He advises to increase the humidity very slowly to avoid forming condensate on the capsules. This avoids the bubble formation from air expelled from the capsule.

After evaluating the suggestions made by Bryonetters, Des Callaghan (Bryonet 3 February 2016) found that to rehydrate the ***Weissia*** capsules (Figure 9), this was the method that worked the best:

1. Put the capsules in a drop of 2% KOH on a glass slide
2. Puncture one side of each capsule (I do this with the tip of very fine forceps)
3. Gently heat the KOH solution with a naked flame beneath the slide

This appears to rehydrate most capsules to their original dimensions, though some can be very stubborn.

Clearing Leaves

I (Glime) have never tried clearing leaves – I wish I had known about this for some of those dirty aquatic species! Des Callaghan (Bryonet 30 August 2012) has demonstrated cleared specimens of ***Anthelia juratzkana*** (Figure 5) in brightfield, phase contrast, and darkfield.

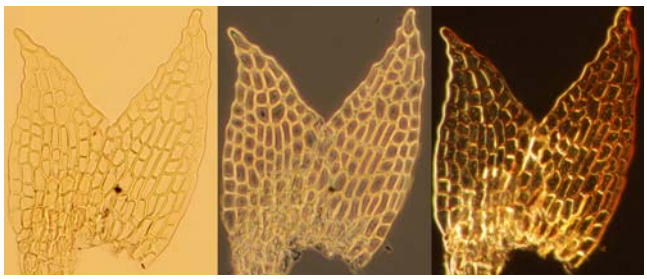


Figure 10. Cleared leaves of ***Anthelia juratzkana***. **left:** brightfield, **mid:** phase contrast, **right:** dark field. Photos by Des Callaghan, with permission.

Lactic Acid

The lactic acid clears all the gunk from the cells, making the walls much easier to see (Rod Seppelt, Bryonet 13 May 2010). Rod Seppelt (Bryonet 13 May 2010) uses lactic acid to clear leaves. One drop on a whole mount is sufficient (particularly if small), or with leaves and sections. The lactic acid may also be added under the cover glass of stems and leaves that have been mounted moist, but not flooded. Gently warm the slide using heat from an incandescent desk lamp. In the lab, if you don't have an incandescent lamp, you can use a hot plate, an alcohol burner, or even a candle, but you will need to clean the carbon off the slide if you wave the slide through the flame or place the slide above the flame. A Bunsen burner is too hot and could result in boiling the solution, a mishap to be avoided!

Unfortunately, lactic acid has its problems. It is somewhat a health hazard if you make contact with it, but less so than phenol, and it is not permanent on the slide. Specimens need to be examined (and drawn if desired) within a few days to weeks.

Water boils more quickly and suddenly than lactic acid, so less water is better. One Bryonetter suggested that a few air bubbles under the cover glass can be a useful **indicator** of imminent danger. When the bubbles begin to expand rapidly, it is time to remove the slide and let it cool so it doesn't boil. If the solution reaches boiling, you will most likely lose most of your dissected leaves and stems as bubbles escape.

KOH or NaOH

Usually these methods will only require a few minutes to clear the specimens. However, for especially dirty ones, you may need to leave the specimen overnight to clear. Potassium hydroxide (KOH) or sodium hydroxide (NaOH) will also clear tissues – particularly if the material is in contact with the air.

Chloral Hydrate

Chloral hydrate works well as a clearing agent, but please read the discussion of its use in Chapter 2-4 of this volume. It is a controlled substance and is dangerous to your health.

If you should choose to use it, the following protocol, developed for clearing parts of the flowering experimental plant *Arabidopsis thaliana*, may be a useful start (Berleth & Jurgens 1993). Substitute solutions for clearing can be tried in place of the chloral hydrate – experiment:

1. Fix plant tissue in 9:1 parts ethanol:acetic acid. Use vacuum infiltration to facilitate penetration of the fix – approximately 2 hours at ambient temperature.
2. Wash tissue twice with 90% ETOH for 30 minutes each wash.
3. Make solution of chloral hydrate or substitute in 30% glycerol. (Note that another substitute might already contain some glycerol.)
4. Add enough clearing agent (chloral hydrate or substitute) to cover the tissue in an Eppendorf tube (ca. 500 mL). Allow tissue to clear several hours.
5. Dissect tissue further if needed, using dissecting microscope. Mount dissected, cleared plant parts in chloral hydrate/glycerol or substitute under coverslip. Seal slide with clear fingernail polish if desired.

Richard Zander (Bryonet 5 March 2017) recommended the use of polyvinyl alcohol-glycerol glue. He originally used a 50:50 mixture, but found it to be too syrupy. He now recommends 70% polyvinyl alcohol glue:30%glycerol. This mix dries to a thick gum. Be generous with this mountant because it is about half water and that will evaporate. Polyvinyl alcohol glue is sold as Elmer's Clear School Glue or Colorations Clear School Glue. The glue also is good for sticking labels to microscope slides. Ready stick on labels dry and fall off in 5-10 years, whereas this glue lasts longer.

Dehydration

Usually specimens are air dried and this is adequate for most species. Some thallose liverworts require preservation, but mosses rarely do. For higher quality specimens, cleaned specimens can be dehydrated with a series of ETOH (70, 90, 100%) (Mayfield *et al*. 1983). Following the dehydration series, specimens are placed in a 1:1 ethyl alcohol:xylene solution, then transferred to 100% xylene. Remove any remaining dislodged soil particles with fine needles. The thalli can then be placed on glass slides in a xylene-soluble mounting medium such as Permount with coverslips that are weighted down with small weights like nuts (of nuts and bolts) or metal washers.

Stains

For most observations, stains are not necessary. But some things are simply too transparent or lack contrast. The series of images of ***Moerckia blyttii*** (Figure 11-Figure 14) by David Wagner illustrate what stains can do to aid visibility of the thallus structure.

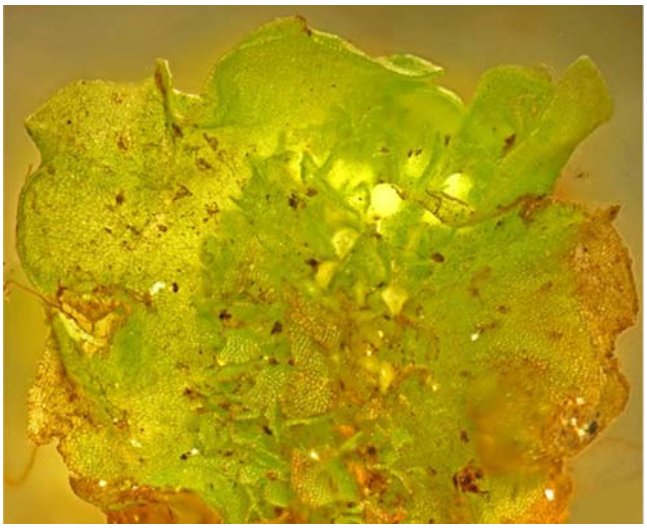


Figure 11. ***Moerckia blyttii*** fresh plant. Photo by David Wagner.

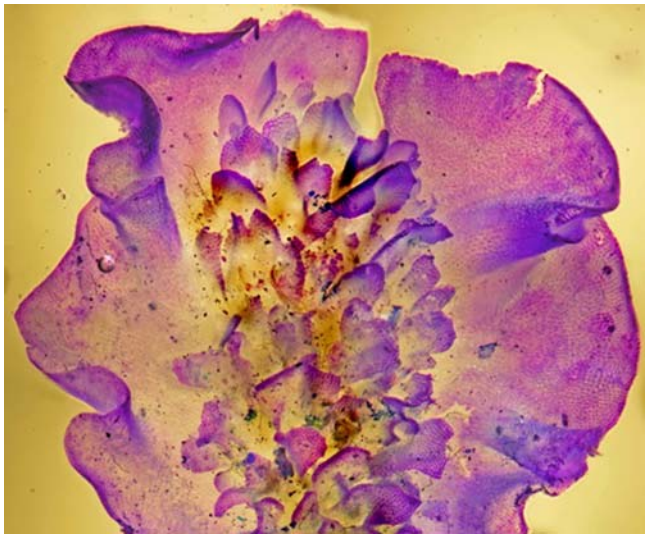


Figure 12. ***Moerckia blyttii*** cleared and stained with methylene blue. Photo by David Wagner.

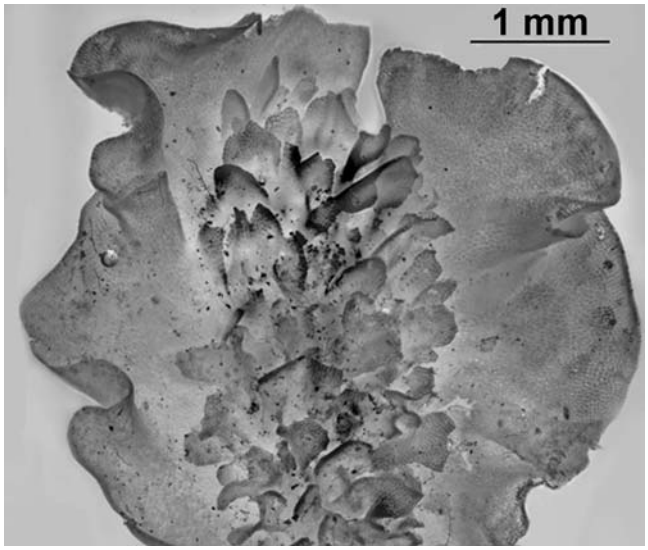


Figure 13. ***Moerckia blyttii*** cleared and stained, grey scale. Photo by David Wagner.

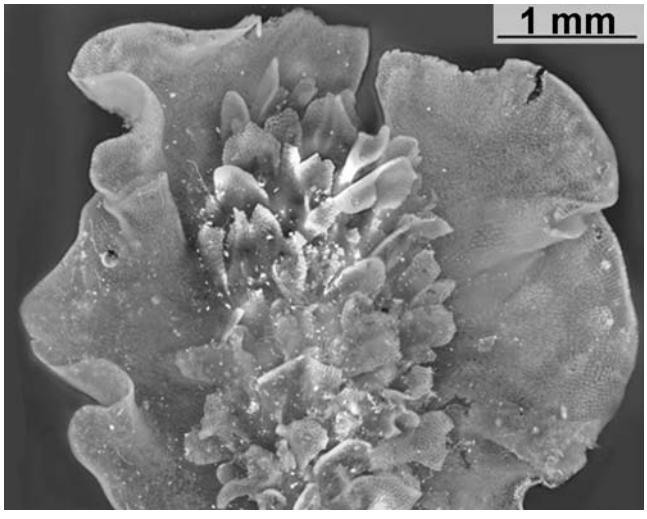


Figure 14. ***Moerckia blyttii*** cleared and stained; gray scale positive image converted to negative. Photo by David Wagner.

Stains can be used for a variety of purposes. They can distinguish cell types, make pores visible, clarify cell walls, make starch visible, and solve other problems in distinguishing special structures. Most stains are readily available, some are toxic, and others are household items.

Tom Thekathyil (Bryonet 13 May 2010) suggests using household chemicals such as those provided by Maier (2012). These include one drop of red or blue **food coloring** in 30 mL of water, or for greater detail and contrast, a mix of one or two drops each of red and blue **food coloring**, five drops water, two to three drops **white vinegar**, and three to five drops **rubbing alcohol**.

Staining Stems

Stems usually have specialized cells, including the epidermis, the cortex, and often a central strand. Others may have hydroids and leptoids and a second distinguishable layer inside the epidermis. In some species, natural colors distinguish the layers, but other specializations may not be easily recognizable.

Triple Stains

Ralf Wagner (pers. comm. 2012) suggests two triple stains that can be used to distinguish cell differences, the Etzold Stain (credited to Dr. Etzold) and W3A. The latter is described (in German) at <http://www.mikroskopie-bonn.de/_downloads/Arbeitsplan_W3Asim.pdf>.

Etzold Stain

Dissolve in 1L water:

Acetic acid (100 %):	20 ml
Fuchsin (bas.)	0.1 g
Chrysoidin	0.143 g
Astralblue	1.25 g

Color Results

non-ligneous cell walls: blue
ligneous cell walls, sclerenchym and xylem: red
Phloem: blue

Kawai Stem Staining Techniques

Kawai did extensive studies on stem sections using a variety of dyes (Kawai 1971a, b, c, 1974, 1975, 1976, 1977a, b, 1978, 1979, 1980a, b, 1981, 1982, 1989, 1991a, b; Kawai & Ochi 1987; Kawai *et al*. 1985, 1986) (Figure 27-Figure 63). He cut stems in 5 or 10 µm, even 15 µm sections (Isawo Kawai, pers. comm. 5 October 1989). Most of the information we have is the result of personal communication and a set of images he sent to me (Glime) many years ago. The effectiveness and time required varied among species and even within a species, perhaps indicating differences in age of the tissue or habitat where it grew.

For his early studies on mosses [**Hypnaceae**, **Thuidiaceae** (Figure 15)], Kawai (1971c, 1975, 1976) rehydrated the mosses by boiling them for half an hour to an hour in water. He then used a standard technique of ethylalcohol-butylalcohol-parafin for fixation. Sections were usually 5 µm thick.

Figure 15. ***Thuidium*** stem with leaves and paraphyllia. Photo by Paul Davison, with permission.

As his work progressed, he experimented with various methods of staining. In early studies, he used **acid fuchsin**, **fuchsin**, **fast green**, and **methyl green** (Figure 25-Figure 27) to stain members of **Bartramiaceae**, **Dicranaceae**, **Entodontaceae**, and **Fissidentaceae** (Kawai 1971). In some cases (**Amblystegiaceae** *sensu lato*, **Dicranaceae**, **Fontinalaceae**, **Hedwigiaceae**, **Lembophyllaceae**, **Leucodontaceae**, **Meteoriaceae**, **Neckeraceae**, **Pterobryaceae**, **Trachypodiaceae**), he used just **gentian violet** and **acid fuchsin** (Kawai 1977b, 1978, 1979). In others (**Amblystegiaceae**, **Bartramiaceae**, **Dicranaceae**, **Hypnaceae**, **Leucobryaceae**) he stained with **gentian violet**, **acid fuchsin**, and **potassium iodide**, using 5 µm sections (Kawai 1980a, b, 1981, 1982). As part of his experimentation with methods, he used 15 µm sections with the **Bryaceae** (Figure 16) (Kawai & Ochi 1987).

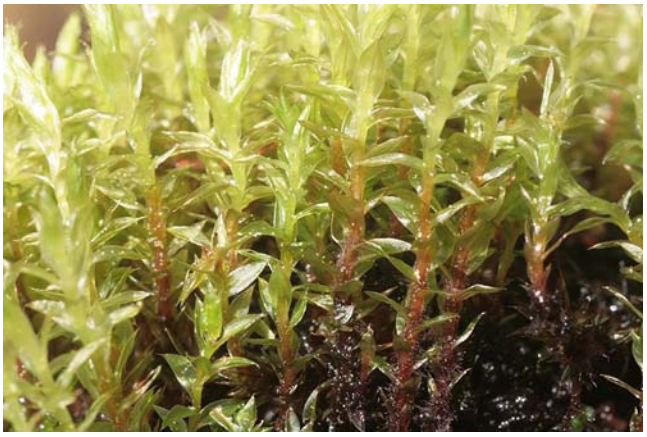


Figure 16. ***Bryum pseudotriquetrum***, a species Kawai cut in 15 µm sections. Photo by Hermann Schachner, through Creative Commons.

Some mosses were much more resistant to the stains. In particular, members of the **Polytrichaceae** (Figure 7) and **Fontinalaceae** (Figure 17) were difficult to stain so that cell types could be seen clearly (Kawai, pers. comm. 5 October 1989). Kawai *et al*. (1985, 1986) ultimately developed a lengthy and more complex protocol that gave satisfactory results. Even this differed between species within the family.

Figure 17. ***Fontinalis antipyretica*** shoot, a family in which Kawai found stems difficult to stain. Photo by Kristian Peters, with permission.

For ***Polytrichum commune*** (Figure 18), Kawai *et al*. (1985) tried three methods. **1) Aniline Blue-Eosin-Methyl Green Method:** They placed the moss in a solution of **aniline blue** and **eosin** for **48 hours**, followed by **washing** and a second solution of just **eosin** for another **48 hours**. Finally, the preparation was **washed** again and placed in a solution of **methyl green** for another **48 hours**. After **washing** the stems were cut in **15 µm** sections with a cryo-microtome and mounted in **gum arabic**. **2) Janus Green-Eosin-Methyl Green Method:** The specimen was soaked in a solution of **Janus green** and **eosin** for **48 hours**, **washed**, and soaked another **48 hours** in just **eosin**. The specimen was **washed** again and soaked in **methyl green** for **48 hours** before the final **washing**, **sectioning**, and **mounting**. **3) Gentian Violet+Congo Red-Eosin-Methyl Green Method:** The specimen started in a solution of **gentian violet** and **Congo red** for **32 hours**. As in the other procedures, it was **washed** and soaked in **eosin**, this time for **40 hours**. Finally it was **washed** and placed in a solution of **methyl green** for **32 hours**, **washed**, **sectioned**, and **mounted**.

Figure 18. ***Polytrichum commune***, a species Kawai used to experiment with stem staining techniques. Photo by Des Callaghan, with permission.

For ***Pogonatum contortum*** (Figure 19), Method 1 was successful, but specimens were soaked in each solution for **32 hours**, except for **40 hours** for just **eosin** (Kawai *et al*. 1985). For ***Rhizogonium*** (Figure 20) and ***Mnium*** (Figure 21), Method 1 was successful, but specimens were soaked in each solution for **72 hours**. For ***Fissidens*** (Figure 22-Figure 23), Method 2 was successful, but specimens were soaked in each solution for **36 hours**. In general, Kawai used the following concentrations:

eosin 0.2 g per 100 cc
methyl green 0.005 g per 100 cc

Figure 19. ***Pogonatum contortum*** with fly taking a drink. Photo from Botany Website, UBC, with permission.

Figure 27-Figure 63 illustrate the responses of a variety of species in various soaking times.

Figure 20. ***Rhizogonium spiniforme*** with capsule, a genus that required soaking in the staining solution for 72 hours. Photo by Janice Glime.

Figure 21. ***Mnium stellare***, a genus that required soaking in the staining solutions for 72 hours. Photo by Des Callaghan, with permission.

Figure 22. ***Fissidens crispus*** shoot, a genus that must be soaked in each staining solution for 36 hours. Photo by Dale A. Zimmerman Herbarium, Western New Mexico University, with permission.

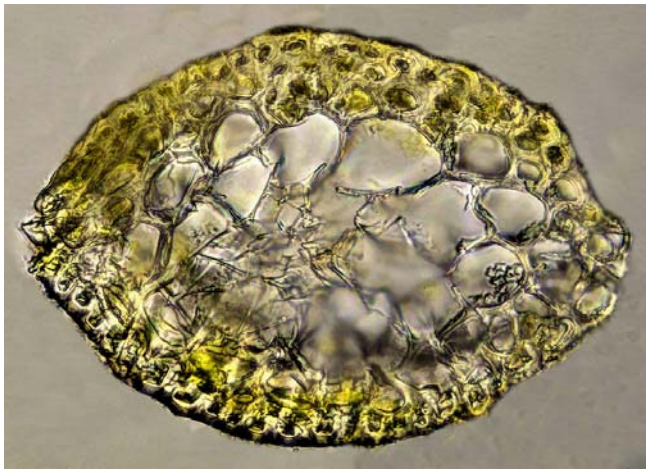


Figure 23. ***Fissidens bryoides*** stem cs, unstained. Photo by Dick Haaksma, with permission.

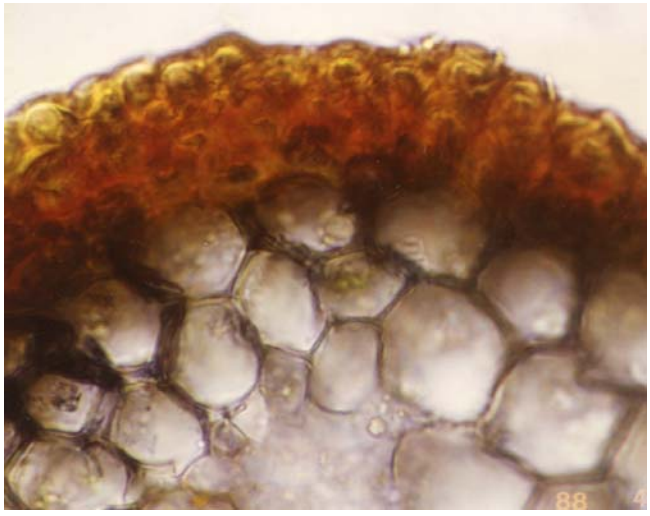


Figure 24. ***Bryoxiphium*** sp. stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

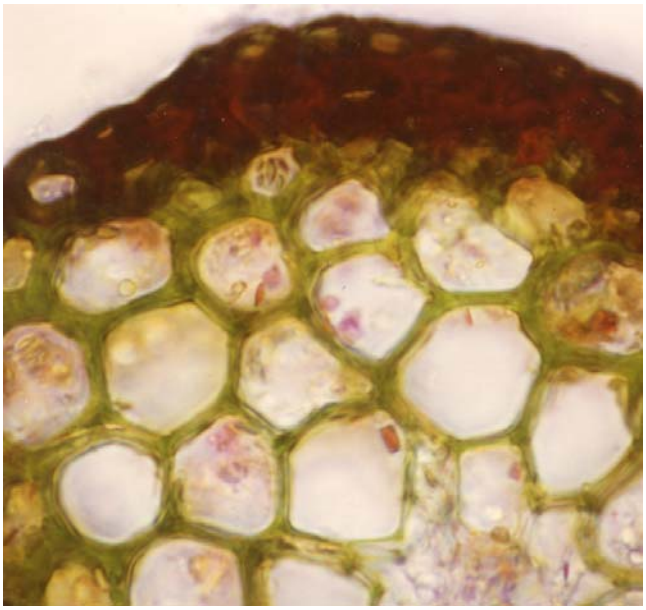


Figure 25. ***Bryoxiphium*** sp. stem cross section stained with **eosin** for 2 hours and **methyl green** for 30 seconds. Photo courtesy of Isawo Kawai.

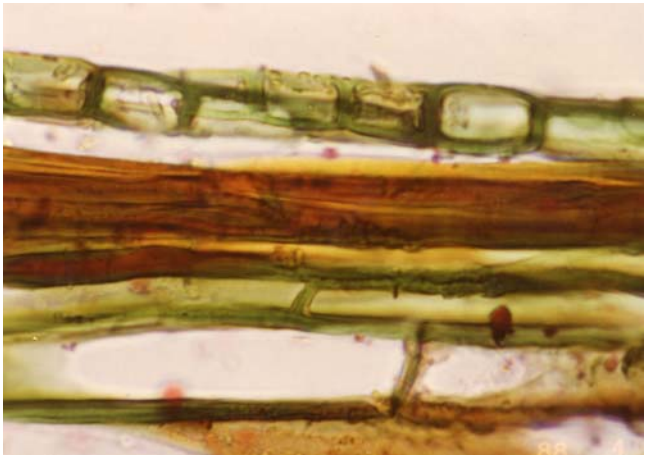


Figure 26. ***Bryoxiphium*** sp. stem longitudinal section stained with **eosin** for 2 hours and **methyl green** for 30 seconds. Photo courtesy of Isawo Kawai.

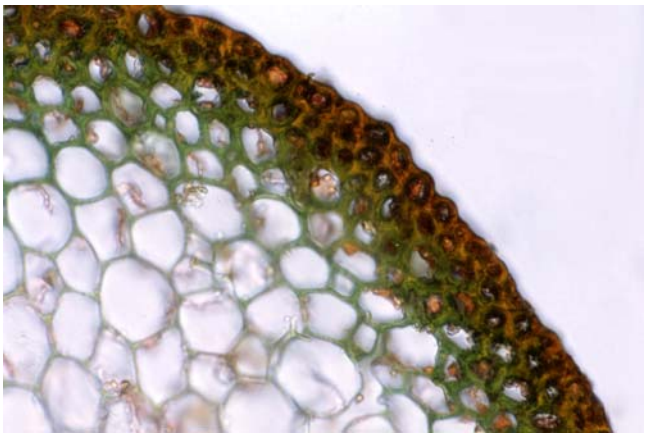


Figure 27. ***Fontinalis antipyretica*** stem cross section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with 0.2 g per 100 cc **eosin** for 50 minutes. The blue-green/green color clearly shows the inner layer of "epidermal" portion of the stem. Photo courtesy of Isawo Kawai.

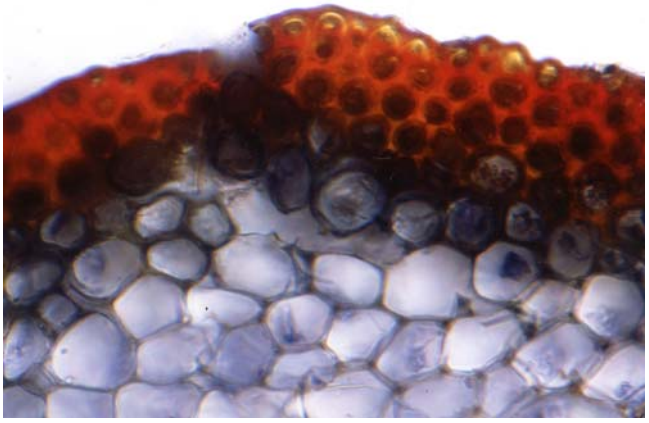


Figure 28. ***Fontinalis antipyretica*** stem cross section stained in **aniline blue** for 20 minutes. Photo courtesy of Isawo Kawai.

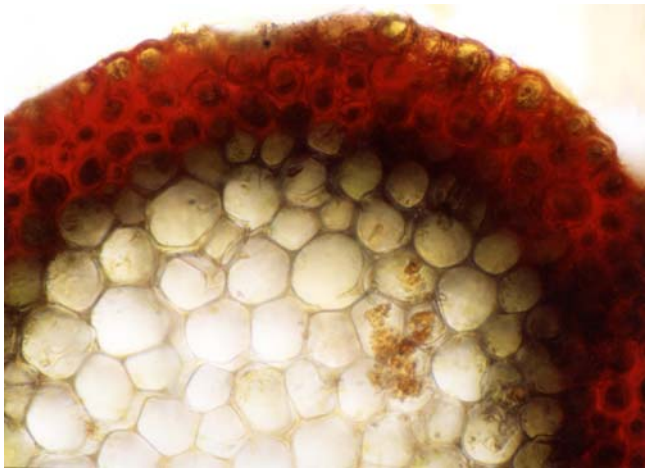


Figure 31. ***Fontinalis antipyretica*** stem cross section stained in **aniline blue** + **eosin** for 1 hour. Compare this to the previous picture (Figure 32) to see differences that can occur under the same staining protocol. These differences may relate to age of the tissues or possibly the habitat. Photo courtesy of Isawo Kawai.

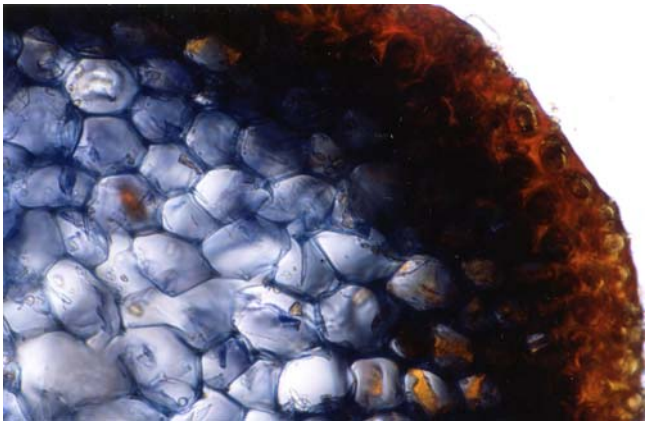


Figure 29. ***Fontinalis antipyretica*** stem cross section stained with **aniline blue** for 30 minutes. Photo courtesy of Isawo Kawai.

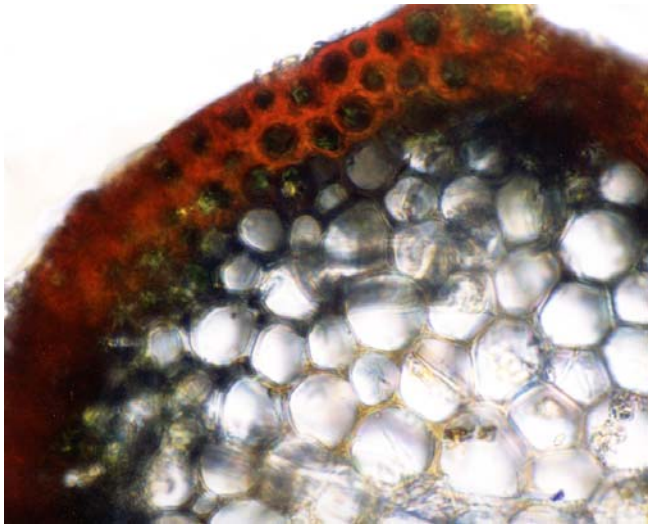


Figure 32. ***Fontinalis antipyretica*** stem cross section stained in **aniline blue** + **eosin** for 1 hour. Photo courtesy of Isawo Kawai.

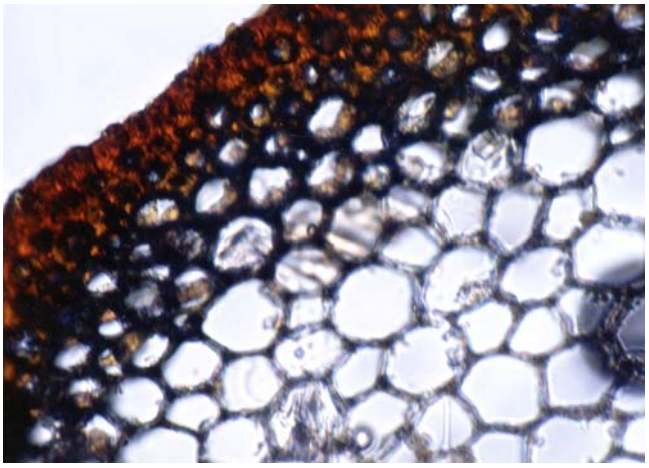


Figure 30. ***Fontinalis antipyretica*** stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

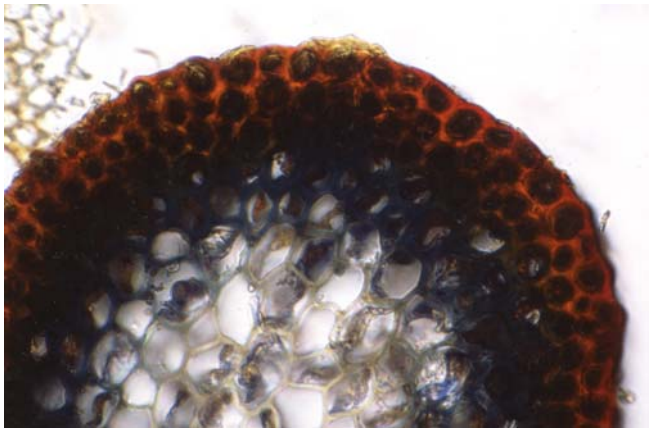


Figure 33. ***Fontinalis gracilis*** stem cross section stained with **aniline blue** for 5 minutes. Photo courtesy of Isawo Kawai.

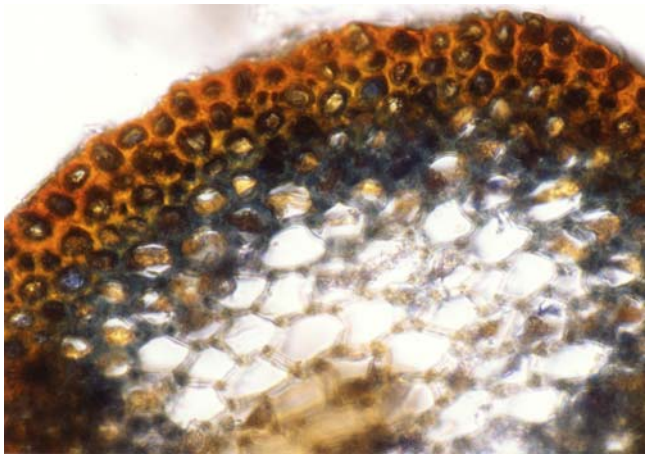


Figure 34. ***Fontinalis gracilis*** stem cross section stained with **aniline blue** for 5 minutes. Photo courtesy of Isawo Kawai.

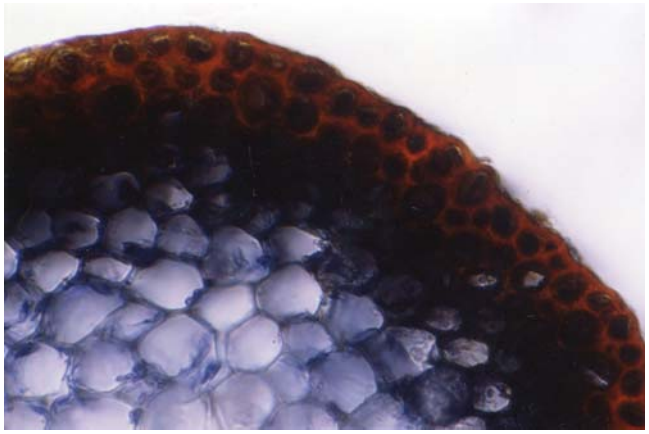


Figure 35. ***Fontinalis gracilis*** stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

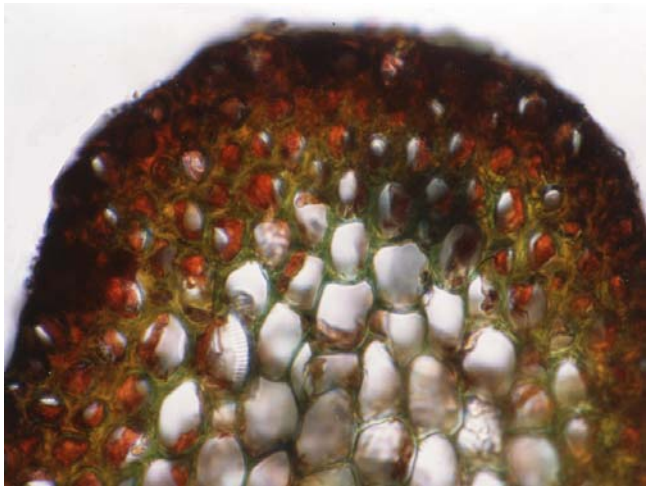


Figure 36. ***Fontinalis gracilis*** stem cross section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with **methyl green** + 0.2 g per 100 cc **eosin** for 15 minutes. Photo courtesy of Isawo Kawai.

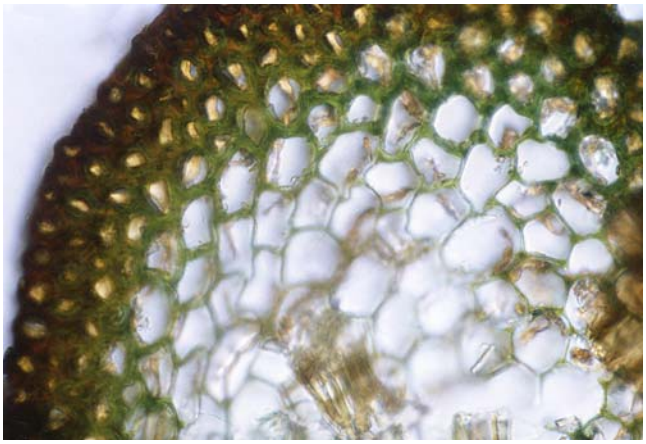


Figure 37. ***Fontinalis gracilis*** stem cross section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with **methyl green** + 0.2 g per 100 cc **eosin** for 1 hour. Photo courtesy of Isawo Kawai.

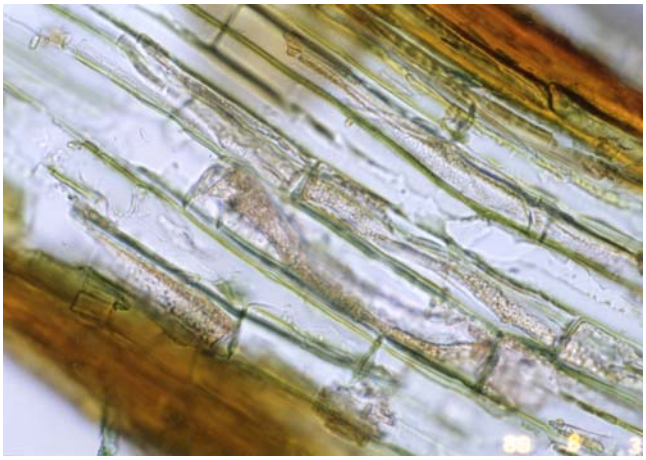


Figure 38. ***Fontinalis gracilis*** stem longitudinal section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with **methyl green** + 0.2 g per 100 cc **eosin** for 15 minutes. Photo courtesy of Isawo Kawai.

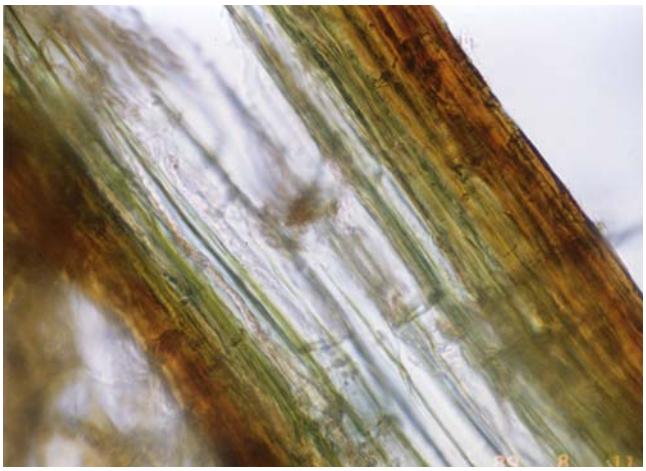


Figure 39. ***Fontinalis gracilis*** stem longitudinal section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with **methyl green** + 0.2 g per 100 cc **eosin** for 1 hour. Photo courtesy of Isawo Kawai.

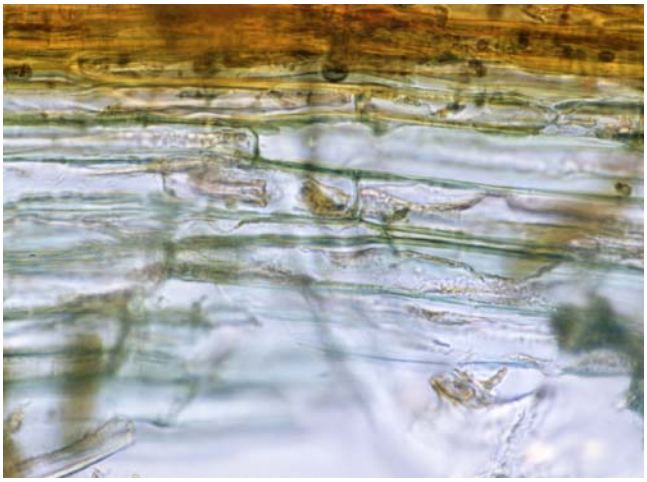


Figure 40. ***Fontinalis gracilis*** stem longitudinal section stained with **eosin** for 1 hour, **washed**, then stained with **methyl green** for 30 seconds. Photo courtesy of Isawo Kawai.

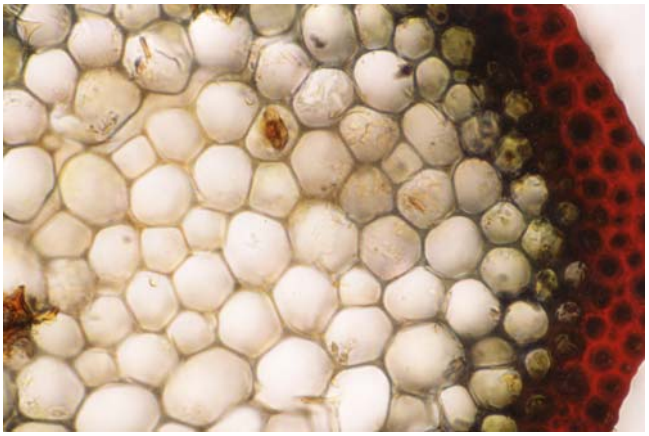


Figure 41. ***Fontinalis hypnoides*** stem cross section stained with **aniline blue** for 30 minutes. Photo courtesy of Isawo Kawai.

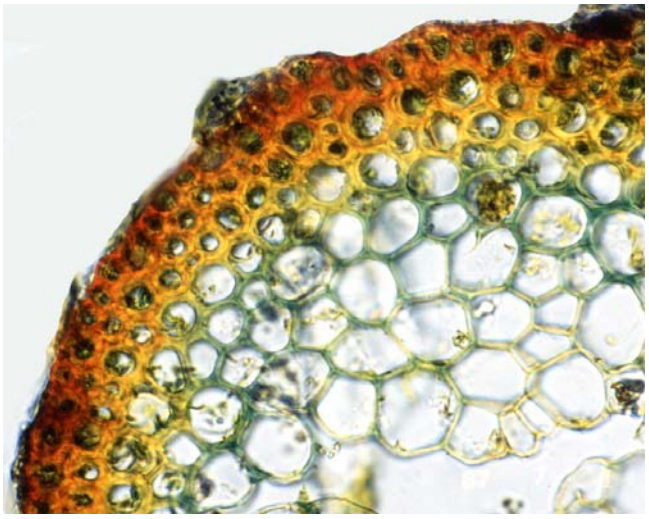


Figure 42. ***Fontinalis hypnoides*** stem cross section stained with **aniline blue** + **eosin** for 3 hours. Photo courtesy of Isawo Kawai.

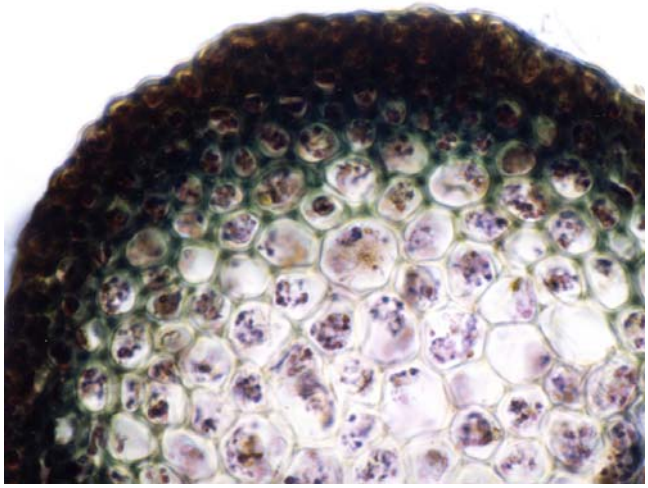


Figure 43. ***Fontinalis hypnoides*** stem cross section stained with **aniline blue** + **eosin** for 7 hours. Photo courtesy of Isawo Kawai.

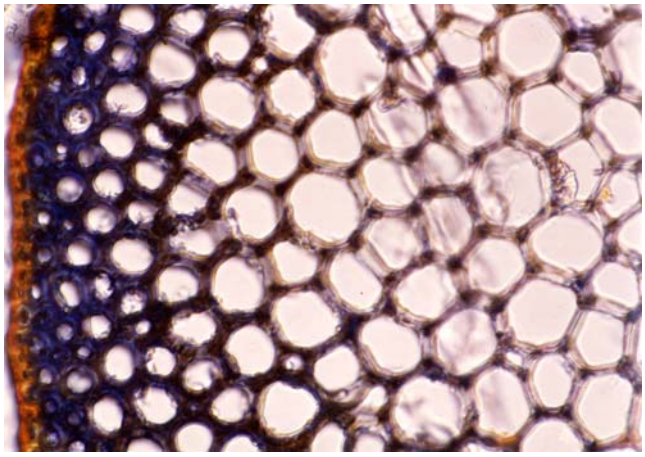


Figure 44. ***Hylocomium*** sp. stem cross section stained with **aniline blue** for 3 hours. Photo courtesy of Isawo Kawai.

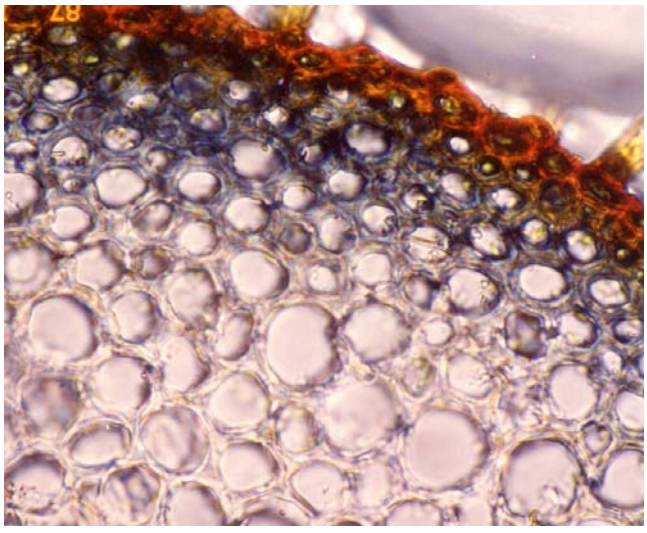


Figure 45. ***Hylocomium*** sp. stem cross section (5 µm thick) stained with **aniline blue** + **eosin** for 2 hours. Photo courtesy of Isawo Kawai.

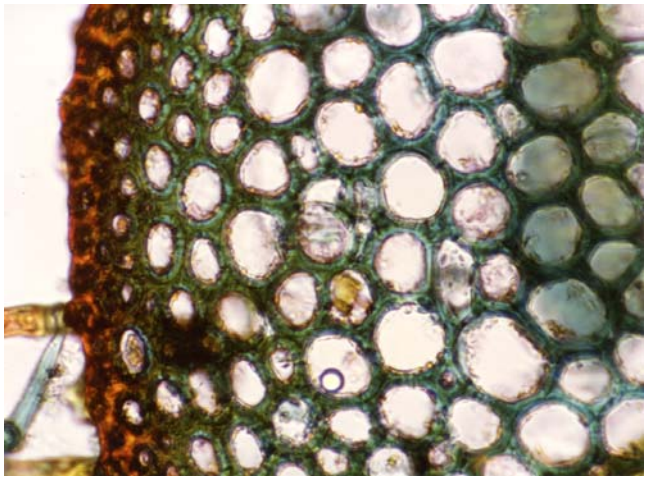


Figure 46. ***Hylocomium*** sp. stem cross section stained with **eosin** for 1 hour, then with **methyl green** 1 minute. Photo courtesy of Isawo Kawai.

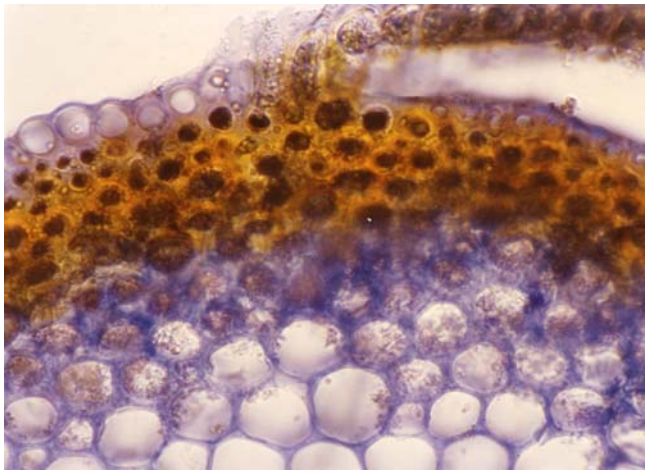


Figure 47. ***Hypnum*** sp. stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

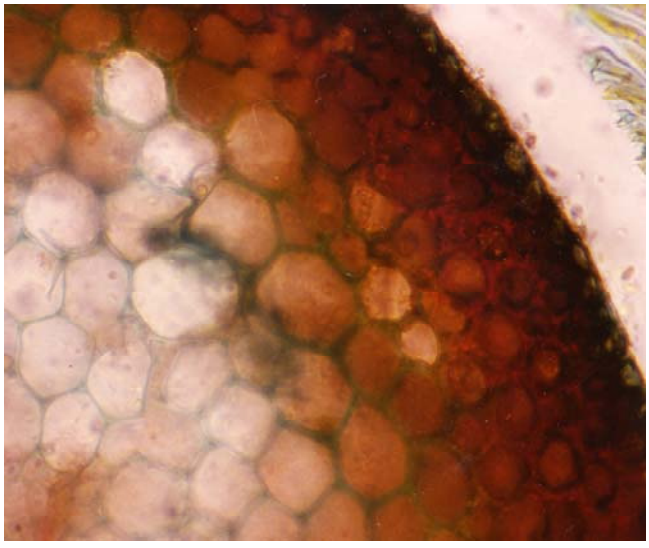


Figure 48. ***Hypnum*** sp. stem cross section stained with **eosin** for 1 hour, **washed**, then stained with **methyl green** for 0.5-1 minutes. Photo courtesy of Isawo Kawai.

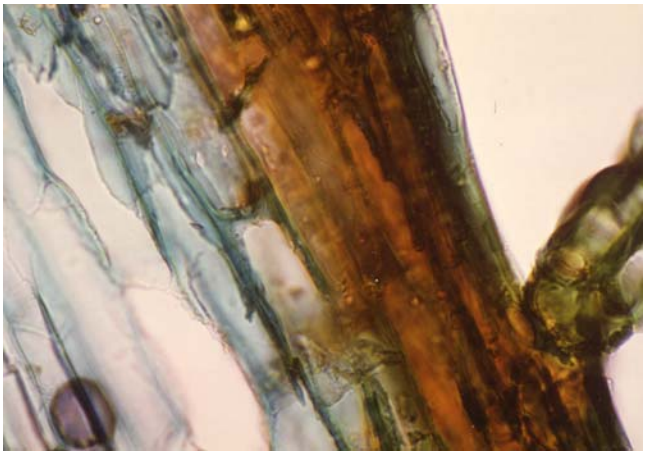


Figure 49. ***Hypnum*** sp. stem longitudinal section stained with **eosin** for 1 hour, then stained with **methyl green** for 30 seconds. Photo courtesy of Isawo Kawai.

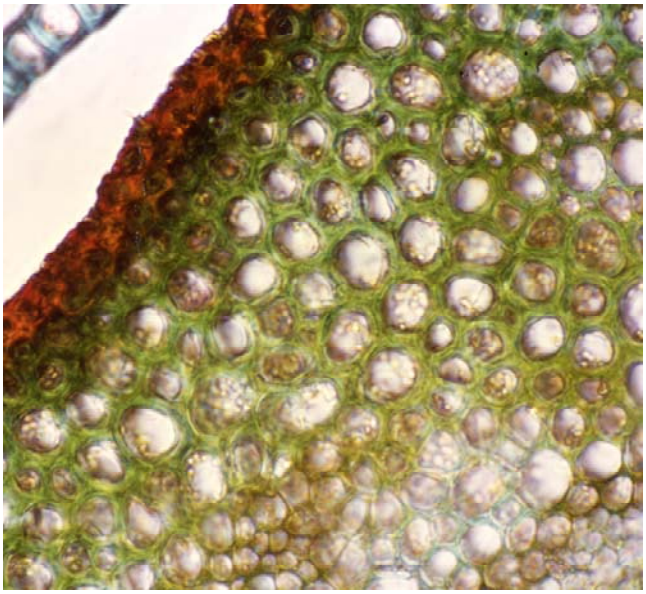


Figure 50. ***Polytrichum*** sp. stem cross section stained with **eosin** for 1 hour, then stained with **methyl green** for 2 minutes. Photo courtesy of Isawo Kawai.

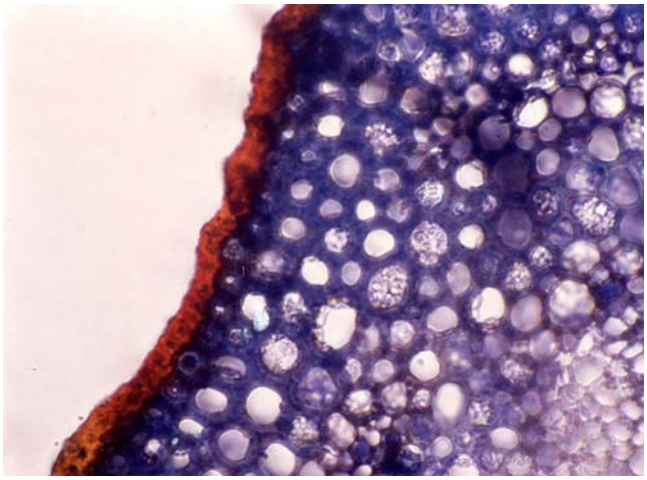


Figure 51. ***Polytrichum*** sp. stem cross section stained with **aniline blue** for 2 hours. Note the cell inclusions in these cortex cells. Photo courtesy of Isawo Kawai.

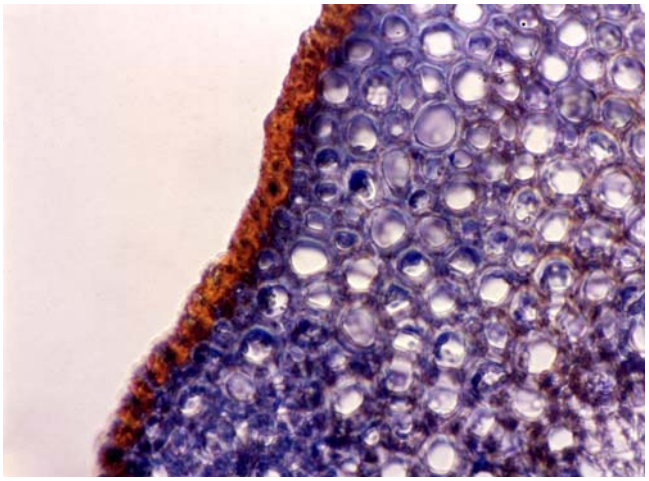


Figure 52. ***Polytrichum*** sp. stem cross section stained with **aniline blue** for 2 hours. Photo courtesy of Isawo Kawai.

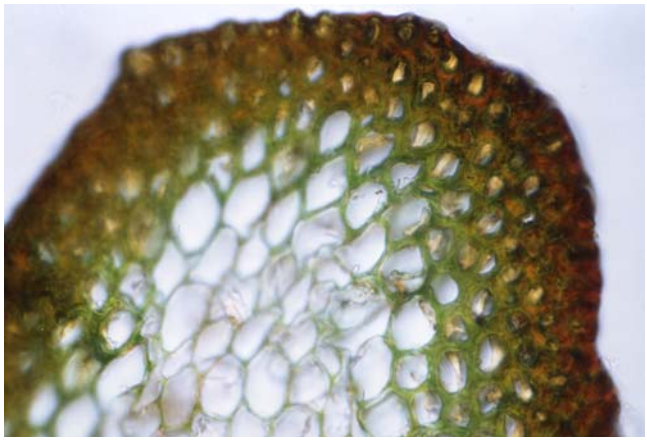


Figure 55. ***Polytrichum*** sp. stem cross section stained with 0.01g per 100 cc **methyl green** for 50 seconds, then stained with 0.3 g per 100 cc **eosin** for 2 hours. Photo courtesy of Isawo Kawai.

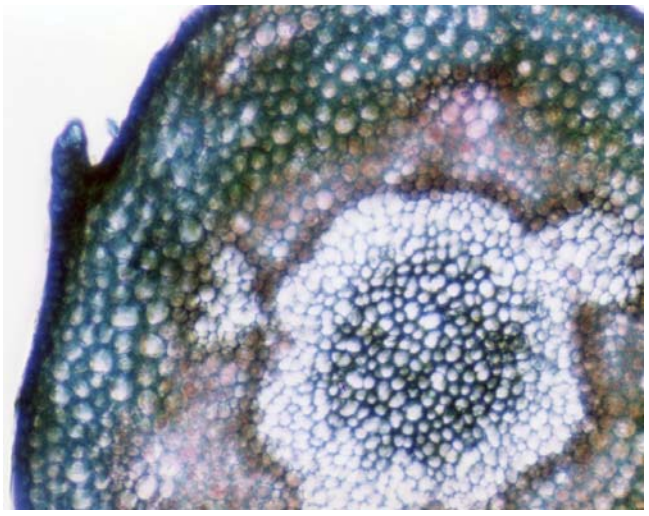


Figure 53. ***Polytrichum*** sp. 10 µm stem cross section stained with 0.01g per 100 cc **methyl green** for 50 seconds, then 0.3 g per 100 cc **eosin** was added for 2 hours, then **washed** with water. Photo courtesy of Isawo Kawai.

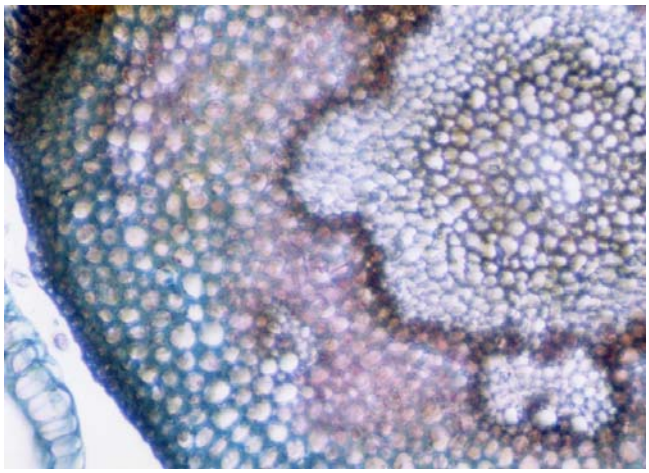


Figure 56. ***Polytrichum*** sp. stem cross section stained with 0.01g per 100 cc **methyl green** for 3 minutes, then stained with **eosin** for 2 hours. Photo courtesy of Isawo Kawai.

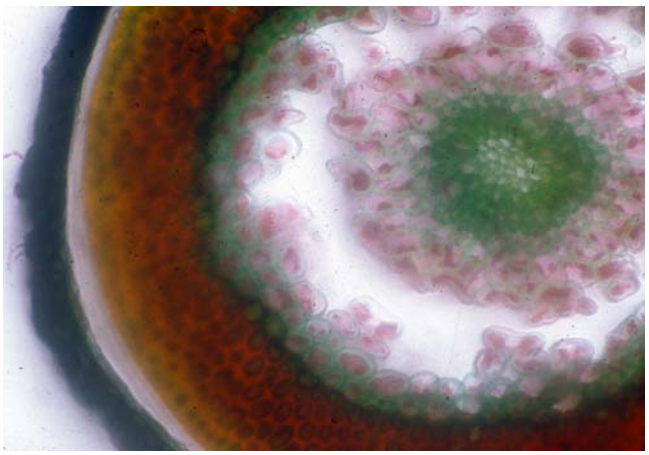


Figure 54. ***Polytrichum*** sp. stem cross section. The cortex cell walls are blue-green from **methyl green**. The hydrome cells are violet-brown. Photo courtesy of Isawo Kawai.

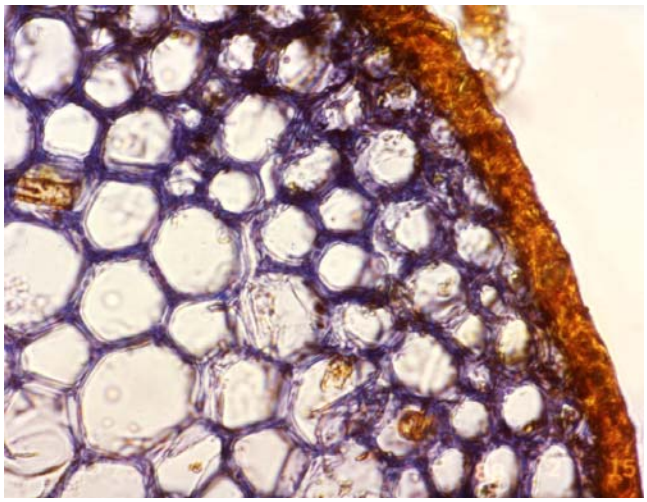


Figure 57. ***Rhizogonium*** sp. stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

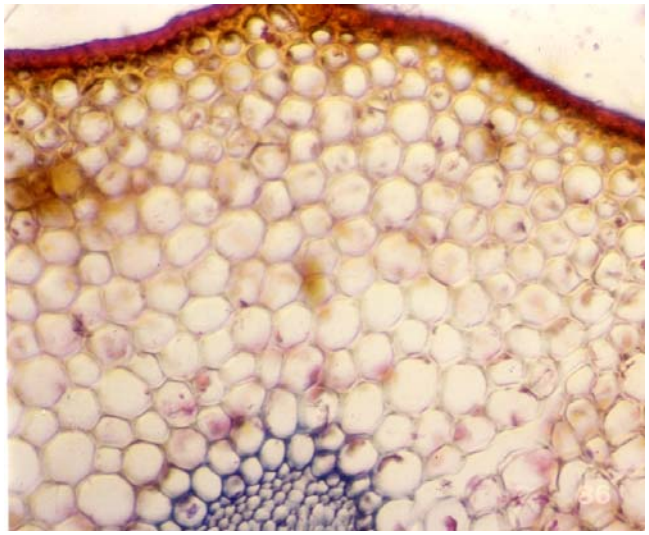


Figure 58. ***Rhizogonium*** sp. stem cross section stained with **aniline blue** + **eosin** for 3 days. Photo courtesy of Isawo Kawai.

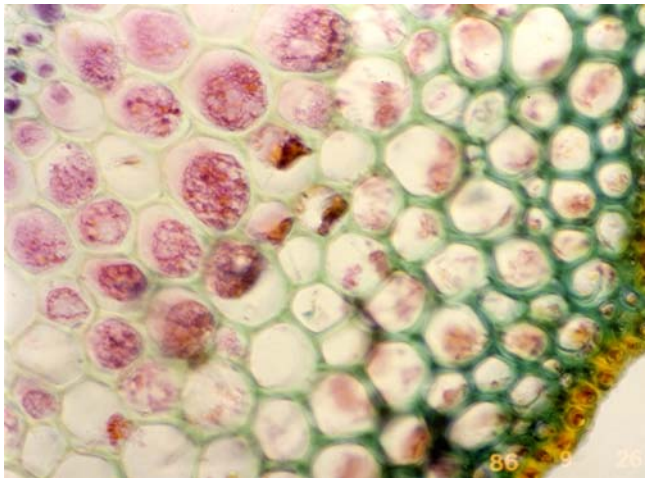


Figure 59. ***Rhizogonium*** sp. stem cross section stained with **aniline blue** + **eosin** for 3 days, washed, stained with **eosin** 3 more days, then stained with **methyl green**. Photo courtesy of Isawo Kawai.

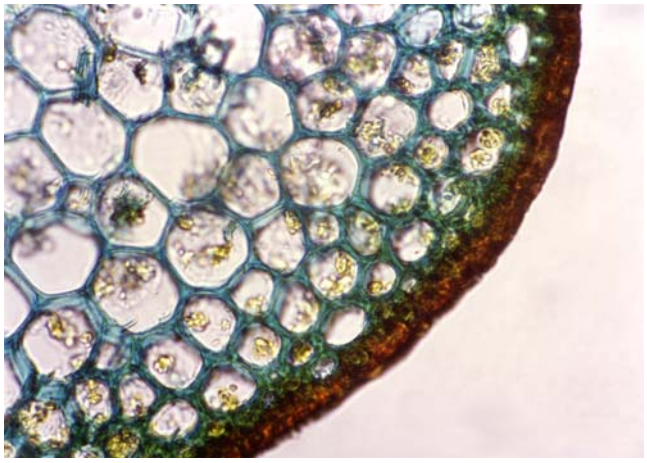


Figure 60. ***Rhizogonium*** sp. stem cross section stained with **eosin** for 2 hours, **washed**, then stained with **methyl green** for 1 minute. Photo courtesy of Isawo Kawai.

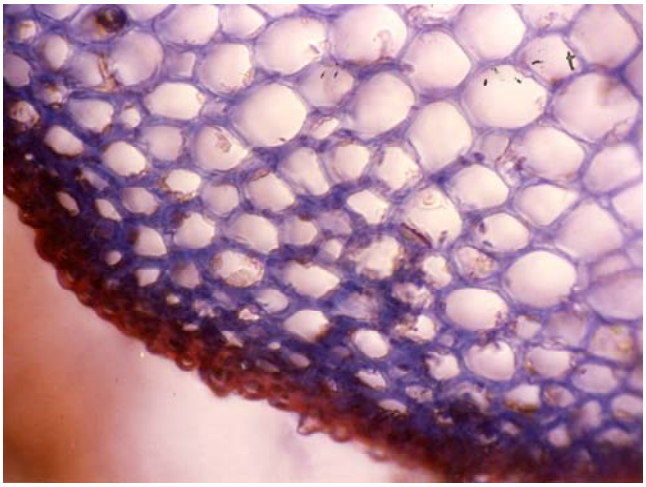


Figure 61. ***Thuidium*** sp. stem cross section stained with **aniline blue** + **eosin** for 2 hours. Photo courtesy of Isawo Kawai.

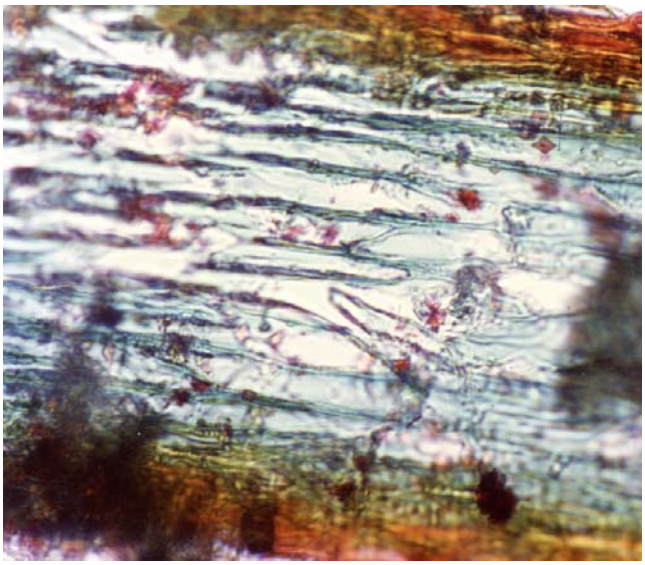


Figure 62. ***Thuidium*** sp. stem longitudinal section stained with **aniline blue** + **eosin** for 2 hours. Photo courtesy of Isawo Kawai.

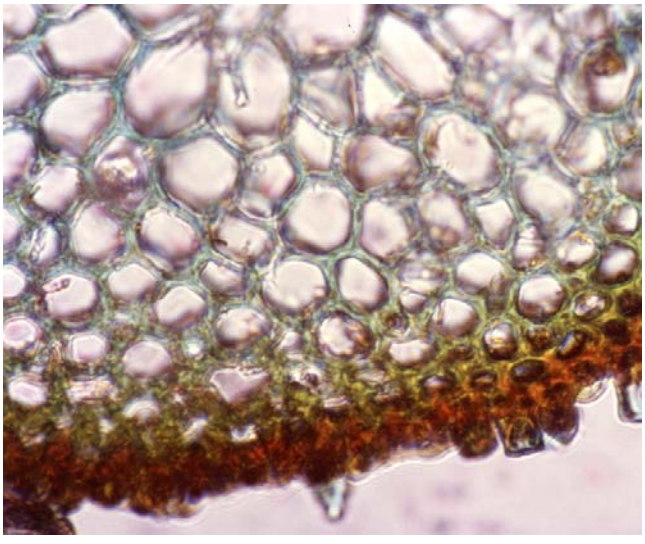


Figure 63. ***Thuidium*** sp. stem cross section stained with **eosin** for 2 hours, washed, then stained with **methyl green** for 1 minute. Photo courtesy of Isawo Kawai.

Acid Fuchsin

Acid fuchsin has been used to stain a variety of plant, animal, and fungal tissues. Kawai (1980b) used **acid fuchsin**, along with **I_2KI** and **gentian violet** to distinguish the internal anatomy of stems in the **Leucobryaceae** (Figure 64). Using 5 µm sections, he was also able to examine the structure in ***Atrichum undulatum*** (Figure 65), ***Bartramia pomiformis*** (Figure 66), ***Dicranum nipponense*** (Figure 67), ***Leucobryum neilgherrense***, and ***Hypnum plumaeforme*** (Figure 68) (Kawai 1981).

Figure 64. ***Leucobryum glaucum***, a genus in which Kawai used acid fuchsin, along with I_2KI and gentian violet to distinguish the internal anatomy of stems. Photo by Janice Glime.

Figure 65. ***Atrichum undulatum*** with capsules, a species in which 5 µm stem sections work well. Photo by Martin Hutten, with permission.

Figure 66. ***Bartramia pomiformis***, a species in which 5 µm stem sections work well. Photo by Ivanov, with permission.

Figure 67. ***Dicranum nipponense*** with capsules, a species in which 5 µm stem sections work well. Photo by Misha Ignatov, with permission.

Figure 68. ***Hypnum plumaeforme***, a species in which 5 µm stem sections work well. Photo by Jan-Peter Frahm, with permission.

Aniline Blue (Kawai & Glime 1988)

Kawai used **aniline blue** to stain several species, including ***Fontinalis*** spp. (Figure 28-Figure 35; pers. comm. 5 July 1988), ***Polytrichum commune*** (Figure 18), and ***Pogonatum contortum*** (Figure 19) (Kawai *et al*. 1985). It stained the epidermal (outermost layers of stem) red and those just inside the red ones were stained blue.

1. Place moss in solution of **aniline blue** and **eosin** for 48 hours. The hydrome cell walls stain violet-brown.
2. After **washing**, place the moss in **eosin** for absorption for 48 hours to stain epidermal cell walls and leptome red.
3. **Wash** again and place moss in solution of **methyl green** for another 48 hours to stain cell walls of cortex blue-green.

Congo Red (Kawai & Glime 1988)

1. Place leafy gametophyte into solution of **gentian violet** and **Congo red** for 48 hours to stain hydrome cell walls violet-brown.
2. **Wash** moss and place in solution of **eosin** for another 48 hours to stain cell walls of epidermis, cytoplasm of leptome, and chloroplasts red.
3. **Wash** again and place moss in solution of **methyl green** for another 48 hours to stain cortex cell walls blue-green.

Eosin

Eosin is a red dye that stains cytoplasm. It is water-soluble and thus can be used to follow water movement through plants. It has been used in the tracheophyte *Arabidopsis* sp. to indicate photodamage to the photosynthetic apparatus (Havaux *et al*. 2000).

Kawai (pers. comm. 8 July 1989) used **eosin** as one of the stains to distinguish cells in ***Fontinalis antipyretica***. This stains the outer cells of the stem ("epidermis") (Figure 27) and the cell walls of the cortex red. **Eosin** likewise stained the cytoplasm of the **leptom** and the chloroplasts red. As noted earlier, this stain works well in most bryophytes to stain cell walls and cytoplasm red.

Fast Green

Fast green is the green dye used in food coloring, but it is known to have tumorogenic effects. It is a protein stain and is one of the stains used by Kawai (1971).

Fuchsin

The dye **fuchsin** is a biological stain that is produced by oxidation of a mixture of **aniline** and **toluidine**, producing a brilliant bluish red. Kawai (1971) used it to stain bryophyte stems.

Gentian Violet (=Crystal Violet)

The color of stain by **gentian violet** depends on the acidity. At *p*H 1.0, the dye is green, but in an alkaline solution it is colorless.

Kawai (1980b) used **gentian violet**, along with **acid fuchsin** and **I_2KI** to distinguish structures within the stems in members of the **Leucobryaceae**.

Janus Green

Janus green is a vital stain that changes color based on the level of oxygen in a cell (Wikipedia 2012). Kawai (pers. comm.) has used it in combination with other stains to stain the **hydrom** of moss stems.

Methyl Green

Isawo Kawai (pers comm. 8 July 1989) used 0.005 g per 100 cc of **methyl green** for 10 seconds to stain cells in 10 µm sections of the stem of ***Fontinalis antipyretica*** (Figure 27). This was followed by **eosin** (0.2 g per 100 cc) added to it. This mix was allowed to stand for 50-60 minutes, then **washed** for observation. **Eosin** stained the outer cells of the stem red and **methyl green** stained those just inside the outermost layers a blue-green color (Figure 27). The central tissue did not stain with this combination.

1. Place leafy moss in solution of **Janus green** and **eosin** for 48 hours to stain hydrome cell walls violet-brown.
2. After **washing**, place moss in solution of **eosin** for further 48 hours to stain cell walls of epidermis, cytoplasm of leptome, and chloroplast red.
3. **Wash** again and place moss in solution of **methyl green** for another 48 hours to stain the cortex cell walls blue-green.

Kawai and coworkers (Kawai *et al*. 1985; Kawai, pers. comm. 5 October 1989) found that the leaf cell walls of ***Polytrichum*** sp. (Figure 54), ***Fissidens*** sp. (Figure 22), and ***Bartramia*** sp. (Figure 66; stained blue-green with **methyl green**, but the cell walls of several species of ***Fontinalis*** (Figure 17) leaves (Kawai, pers. comm. 5 October 1989) would not stain with methyl green.

Leaves

I_2KI – Lugol's Solution (Kruijer & Klazenga 1994)

Kruijer and Klazenga (1994) consider **methylene blue,** a common ***Sphagnum*** (Figure 69) stain (see chapter on *Sphagnum* Staining in this volume), to be somewhat problematic for other leaves, sometimes staining too darkly. Instead, they recommend staining with a diluted solution of **iodine-potassium iodide** (I_2KI), or Lugol's solution (Johansen 1940). This is the well known stain for starch, causing it to turn blue to purple to nearly black. But it can also stain **cellulose** if tissues are first hydrolyzed with sulfuric acid and **hemicellulose** if hydrolyzed with hydrochloric acid. Kruijer and Klazenga used **I_2KI** successfully on leaves and cross sections of members of the **Hypopterygiaceae** (Figure 70) and the genus ***Dicranoloma*** (Figure 71). Cell walls became brighter, but remained nearly colorless except for the middle lamella, which sometimes became bright yellow.

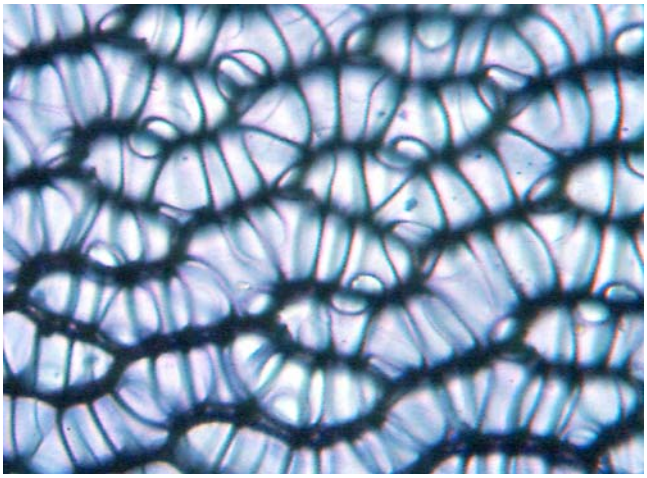


Figure 69. ***Sphagnum*** leaf cells stained with methylene blue to make pores visible. Photo by Janice Glime.

Figure 70. ***Canalohypopterygium filiculaeforme***. Some members of its family (**Hypopterygiaceae**) can be stained with I_2KI. Photo by Jan-Peter Frahm, with permission.

KOH (Zander 1989, 1993)

KOH in concentrations from 2% to saturated will stain cell walls of many mosses. It can be used on whole leaf mounts and on sections. In the **Pottiaceae** the resulting colors can be used as diagnostic characters. Zander (1993) uses it to rehydrate mosses as well. The **KOH** should not be kept in glass dropper bottles because it reacts with the glass to form a precipitate. If the specimen will later be mounted with an acidic mountant, add a drop or two of dilute **HCl** to the specimen.

Figure 71. ***Dicranoloma billardieri***. Some members of its genus can be stained with I_2KI. Photo by Michael Lüth, with permission.

KOH is useful in distinguishing between genera in the **Pottiaceae** (Zander 1993). For example, the lamina color reaction in ***Tortula*** (Figure 72) and ***Ganguleea*** is yellow, whereas in ***Syntrichia*** (Figure 73), ***Dolotortula***, ***Chenia*** (Figure 74), ***Hilpertia*** (Figure 75), ***Sagenotortula*** (Figure 76), ***Stonea***, and ***Hennediella*** (Figure 77) it is red, and in ***Saitobryum*** (Figure 78), deep red (Zander 1989).

Figure 72. ***Tortula intermedia***, in a genus that stains yellow in KOH. Photo by Michael Lüth, with permission.

Figure 73. ***Syntrichia ruralis***, in a genus that stains red in KOH. Photo by Michael Lüth, with permission.

Figure 74. ***Chenia leptophylla*** in arable field, in a genus that stains red in KOH. Photo by Jonathan Sleath, with permission.

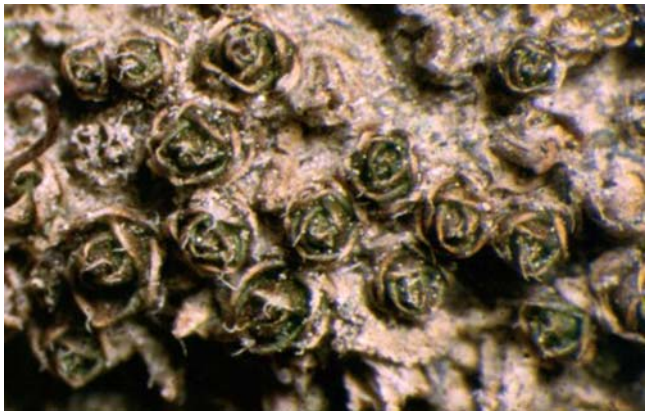


Figure 75. ***Hilpertia velenovskyi***, in a genus that stains red in KOH. Photo by Jan-Peter Frahm, with permission.

Figure 76. ***Sagenotortula quitoensis***, in a genus that stains red in KOH. Photo by Claudio Delgadillo Moya, with permission.

Figure 77. ***Hennediella stanfordensis***, in a genus that stains red in KOH. Photo by Paul Wilson, with permission.

Figure 78. ***Saitobryum peruvianum***, in a genus that stains deep red in KOH. Photo by Claudio Delgadillo Moya, with permission.

Safranin O / Fast Green (Rod Seppelt, Bryonet 15 August 2012)

Rod Seppelt (Bryonet 15 August 2012) considers this a good general stain for plant sections. It works well for bryophytes on specimens that have been fixed and embedded and on sectioned material. Bill and Nancy Malcolm (2006) have used this combination to obtain high-contrast color effects. The technique is somewhat time-consuming, requiring a schedule of dehydration and rehydration. They suggest a quicker option using **toluidine blue**. If it is used simply to clear the cells, then the hydrolyzation step is unnecessary.

Lisa Op den Kamp (Bryonet 4 October 2012) also uses **safranin**. She applies this directly to the leaves or capitula of ***Sphagnum*** (Figure 79), then washes them in water, all before cutting the ***Sphagnum*** to make the desired sections. Safranin normally dyes lignin red; although ***Sphagnum*** doesn't have typical lignin, safranin stains the lignin-like compounds in the tissues. She has kept the solution for 12 years and it still works well.

Figure 79. ***Sphagnum auriculatum*** capitula, not stained. Photo by Jan-Peter Frahm, with permission.

Sphagnum Stains

In particular, ***Sphagnum*** (Figure 69) leaves typically need to be stained for the pores to be visible. Rudi Zielman (Bryonet 4 October 2012) considers there to be four ***Sphagnum*** stains (see Chapter 2b for details):

aniline blue
methylene blue
gentian violet (=crystal violet)
toluidine blue O

These can be applied in two ways: supply a bit of it directly in a few drops of alcohol or water or make a stock solution based on alcohol or water.

To enhance the pores on ***Sphagnum*** (Figure 80) leaves, Rod Seppelt (Bryonet 13 May 2010) suggests **toluidine blue**, **aniline blue**, or **methylene blue**. A drop or two in 100 ml of water should be sufficient. Schofield (1985) recommends **methylene blue**, **gentian violet**, or **crystal violet** in a 1-2% aqueous solution. If the stain is very dark, simply dip the moss in quickly and then rinse it in clear water. If it gets too much stain, you will see even less than with no stain. Be careful – these stains also stain fingers and clothing! If you don't have the standard stains, try experimenting. We wonder if beet juice would work. It might need a bit of vinegar to make it colorfast for permanent mounts.

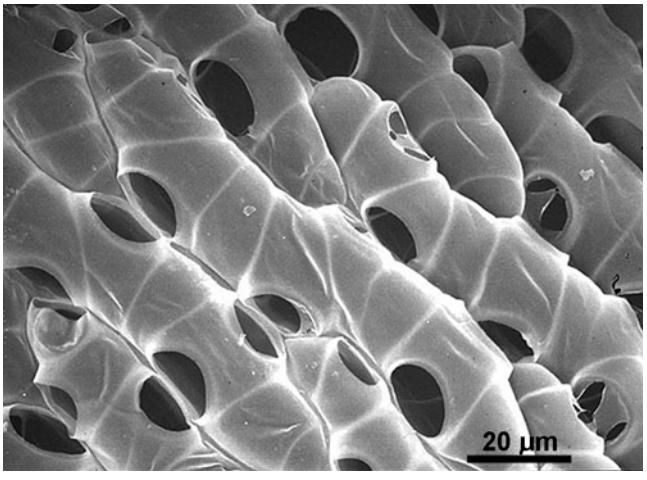


Figure 80. SEM of ***Sphagnum*** hyaline cells and pores. Photo from Botany Website, UBC, with permission.

Methylene Blue (Kruijer & Klazenga 1994; Wagner, Bryonet 11 May 2010)

When staining ***Sphagnum*** (Figure 69) pores, it is important not to stain too heavily. Kruijer and Klazenga (1994) use a 1-2% aqueous solution of **methylene blue**. Or, place a drop of full strength dye on a slide or in a Syracuse watch glass. Dip the ***Sphagnum*** branch quickly into the dye to cover the branch, then dip the branch into clean water to wash the dye off. Don't allow the branch to remain in the dye. After washing, the moss should be ready for viewing.

David Wagner (Bryonet 11 May 2010) brings us a simple solution for staining ***Sphagnum*** (Figure 69), a contribution from one of his students. Since **methylene blue** is used as an antibiotic for aquarium fish for hatching eggs or getting rid of fungal infections, it is readily available at tropical fish stores. A half ounce bottle (ca 12 ml) of VERY concentrated **methylene blue** is only about US $4.25 and will be a lifetime supply.

Crystal Violet/Gentian Violet

Crystal violet, also known as gentian violet or methyl violet 10B, is the compound hexamethyl pararosaniline chloride, or pyoctanin(e), and is a triarylmethane dye.

Adam Hölzer (Bryonet 4 October 2012) reports that he can see even the pores of ***Sphagnum obtusum*** (Figure 81) very well with **crystal violet**. He dissolves some powder in about 50 ml of distilled water with the addition of some alcohol to preserve it. He adds new alcohol occasionally to compensate for evaporation. He puts the moss leaves in a drop of water. Then uses his forceps to dip into the solution and transfer only a small drop into the drop of water. He covers the drop with a cover glass. The color stains the cellulose of the leaves Excess stain can be removed by adding water to one side and drawing off the solution on the other with tissue paper. The 50 ml of solution will last for several years even if you use it every day. Stains on the desk can be cleaned with alcohol.

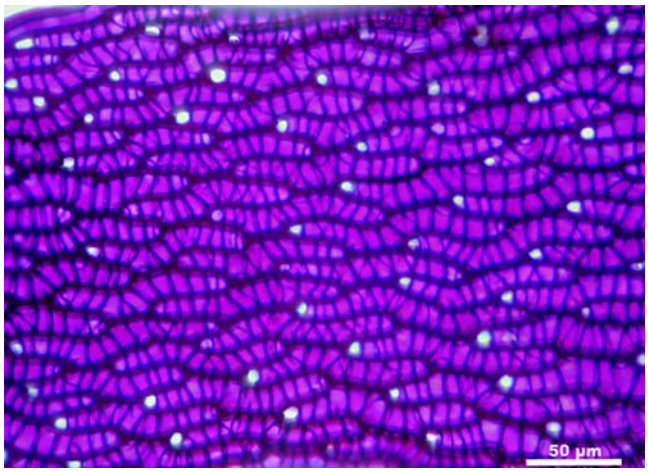


Figure 81. ***Sphagnum obtusum*** stained for pores. Photo by Ralf Wagner.

Crystal violet and gentian violet solutions can be used to fill well-rinsed and dried felt-tip pens (Joannes (Jan) A. Janssens, Bryonet 4 October 2012). These pens can be used in the field to stain ***Sphagnum*** (Figure 81) that has been squeezed somewhat dry.

Toluidine Blue O (Rod Seppelt, Bryonet 15 August 2012)

Rod Seppelt (Bryonet 15 August 2012) considers this to be the most useful stain for general tissue differentiation in fresh material, but it is not useful for permanent mounts. It can help to distinguish the ventral row of leaves in **liverworts**. It also will reveal the pores and stem leaves in ***Sphagnum*** (Figure 81).

Simple method:

0.2%-0.25% toluidine blue O in water (be sure it is O), or 1 drop in 10 drops of water

Stain moss in solution for 10-30 seconds, place on slide, apply cover glass, and examine (without washing excess stain away). If too dark, dilute the stain further before use, or wash the moss quickly to remove some of the excess.

In vascular plants, its multiple color responses can indicate tissue type: phloem green, xylem blue, parenchyma purple, lignified tissue of bundle caps pale whitish-green. Similar color distinctions may occur in bryophytes. Unfortunately, the color is not permanent.

More complex recipe:

0.610 g KH_2PO_4
0.970 g K_2HPO_4
0.050 g Toluidine Blue O
In 100 ml distilled water

Des Callaghan (Bryonet 4 October 2012) likewise recommends Toluidine Blue O and Safranin O for ***Sphagnum*** (Figure 81). Simply dip the branch in the stain and it works almost immediately. You can find the stains on eBay cheaply (*e.g.* items 261098492176 and 261107216623). But he cautions that for non-aqueous permanent mounts, these stains are not suitable. Instead, Bismark Brown provides a nice stain.

Reproductive Structures

Iron Haematoxylon / Fast Green (Rod Seppelt, Bryonet 15 August 2012)

This stain works very well to show archegonia and spermatogenous cells in antheridia (Rod Seppelt, Bryonet 15 August 2012).

Bulbils and Spores

Fluorescence and Fluorescent Dyes (Nordhorn-Richter 1988)

Gisela Nordhorn-Richter (pers. comm.) discovered the fluorescence of bulbils in ***Pohlia*** (Figure 82-Figure 83) when a microscope salesman visited her institution. No one was visiting the display and she felt sorry for the salesman, so she took some of her specimens to look at them. She was amazed at the ease of finding bulbils with the fluorescence technique.

Figure 82. ***Pohlia bulbifera*** with bulbils that can be located by their fluorescence. Photo by Misha Ignatov.

Figure 83. ***Pohlia bulbifera*** bulbils that can be located by their fluorescence. Photo by Des Callaghan, with permission.

Preparation of bryophytes for fluorescence microscopy is mostly what not to do. They can be prepared on a slide with water or as permanent slides (Nordhorn-Richter 1988). However, some of the embedding materials have phenolic compounds as preservatives or may have a synthetic resin. These produce fluorescence that interferes with seeing the bryophyte structures. Air bubbles are another potential problem because they can scatter the light. Dry plants can only be rewet once because the membranes typically are destroyed by drying. When the plants are rewet, water soluble substances leak from the cell. When they dry once again, the water-soluble fluorescing substances disappear, ending fluorescence.

In the dried condition, fluorescing substances of bryophytes are very stable, with rhizoid bulbils (Figure 84) of ***Pohlia*** that are more than 100 years old still exhibiting brilliant fluorescence. Chlorophyll, on the other hand, loses its fluorescent ability upon drying.

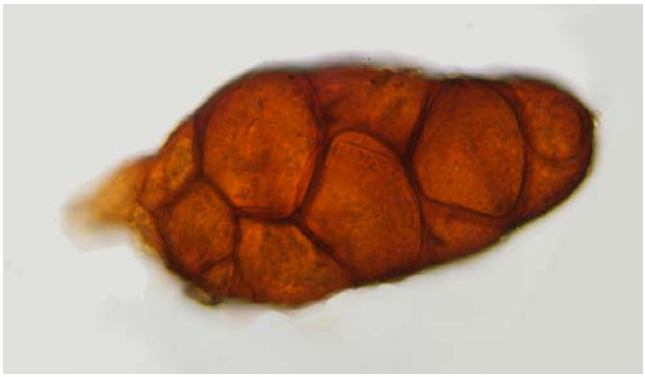


Figure 84. ***Pohlia wilsonii*** rhizoidal tubers. Photo by Guillermo M. Suárez, with permission.

To hide the fluorescence of chlorophyll, which can interfere with fluorescence of other substances, a suppression filter of 650 nm can absorb its red fluorescence (Nordhorn-Richter 1984a, b, 1985a, b, 1988). Alternatively, the chlorophyll can be extracted by 80% acetone or DMSO without interfering with other fluorescent substances.

The fluorescence technique for bryophytes permits one to find rhizoid gemmae hiding in a sandy substrate (Nordhorn-Richter 1988). Live spores exhibit red fluorescence, permitting estimation of vitality that can be quantified with a fluorescence spectrophotometer (Figure 85; Ridgway & Larson 1966; Paolillo & Kass 1973; Genkel & Shelamova 1981). Phenolic acids, including ***Sphagnum*** acid (Tutschek 1975), lignin-like compounds in cell walls (Lal & Chauhan 1982; Nordhorn-Richter 1984a, 1985), peristome structure (Nordhorn-Richter 1985b), and papillae (Nordhorn-Richter 1984b) become visible. Even small bryophytes can be found by using a UV light (366 nm) at night! (Nordhorn-Richter 1983). Gambardella *et al*. (1993) used fluorescence microscopy to examine the cytoskeleton of the columella in ***Timmiella barbuloides*** (Figure 86). Animal tissues exhibit only secondary fluorescence, making it possible to distinguish between animal galls and bryophyte propagules (Nordhorn-Richter 1988).

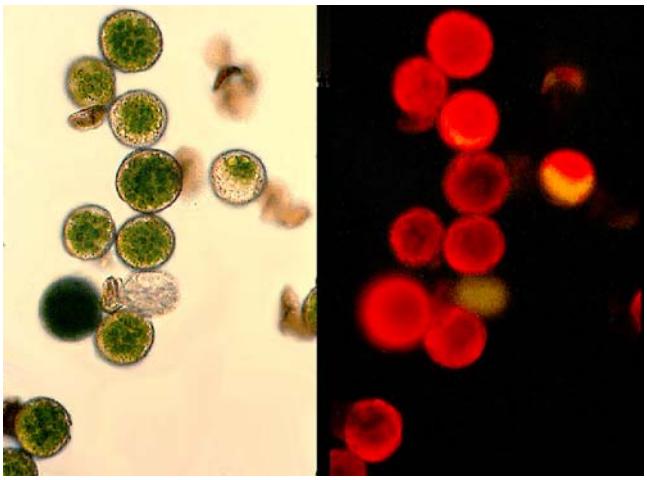


Figure 85. Spores of ***Fontinalis squamosa*** showing spores in white light on left and fluorescing under UV light on right. Note that the living spores show up as red under fluorescence, whereas dying and dead spores are yellow or invisible. Photos by Janice Glime.

Figure 86. ***Timmiella barbuloides***, the species used by Gambardella and coworkers to examine the cytoskeleton with fluorescence. Photo by Jonathan Sleath, with permission.

Shellhorn *et al*. (1964) demonstrated that both fresh and fossil pollen could be detected with **fluorochromes**, with better results if **acridine orange** was added to enhance detail. Ridgway and Larson (1966) extended the fluorescence technique to provide better viewing of the features of the hornwort ***Anthoceros*** (Figure 87). The images of spores of ***Fontinalis squamosa*** demonstrate that the use of fluorescence microscopy can help to distinguish living from dead spores in mosses (Figure 85). The yellow fluorescence in the image suggests that the exine is fluorescing, as it does in pollen (Ridgway & Larson 1966).

Figure 87. ***Anthoceros agrestis***, a hornwort in which fluorescence microscopy helps to reveal its structures. Photo by Bernd H through Creative Commons.

Stains can provide one with the ability to see structures using fluorescence microscopy. Brandes (1967) explained the use of **acridine orange** as a vital stain for use with fluorescence microscopy of protonemal pro-buds and buds. The stain moves to the cytoplasm, combining with the RNA. This technique shows the increase of cytoplasmic RNA immediately after the induction of the pro-buds. Hence, kinetin-induced buds, as well as non-induced branches, can be detected ten hours after the beginning of a kinetin treatment.

Fluorescent dyes can have various purposes, including using them as growth markers in the field (Russell 1988). **Fluorochrome** 3,3'Dihexyloxacarbocyanine iodine [$DiOC_6(3)$] can be used to locate selectively the fungal hyphae among the rhizoids of bryophytes (Duckett & Read 1991). Ascomycetous hyphae are visible when concentrations of 0.01-5 µg ml^{-1} are used, whereas to see Basidiomycetes that form endophytic associations, the concentration needs to be at least 50 µg ml^{-1}. Some fungi, such as VA fungi in liverworts, do not stain with **fluorochrome** at any concentration. Others require a much lower concentration than these. One advantage to this method is to recognize the extent of the fungal hyphae in the association.

Staining Liverwort Capsules (Von Konrat *et al*. 1999)

Von Konrat *et al*. (1999) devised a technique to examine the multiple layers of the capsule wall of liverworts. First the layers need to be separated using a **pectinase** preparation. Then the layers need to be cleared and stained to make the details easier to see.

They recommended doing all the treatments on the same slide – results were less satisfactory when the specimen was moved from one reagent to another. Solutions can be removed between treatments by using filter paper cut into strips. The capsule was mounted on a coverslip and the fully stained capsule was mounted between two coverslips so that both surfaces could be examined.

1. Treat with FAA for 24 hours or until decolored

FAA (Formalin-Acetic-Alcohol)	**(100 ml)**
Ethyl alcohol	50 ml
Glacial acetic acid	5 ml
Formaldehyde (37-40%)	10 ml
Distilled H_2O	35 ml

2. **Rinse** in water three times.
3. Clear partially with 80% **lactic acid** at 60°C for 30-60 minutes in container saturated with water vapor.
4. **Wash** again at least three times in water.
5. Add enough 1% (v/v) **pectinase** preparation of *Aspergillus niger* in water to cover specimen. Let stand for a maximum of 1 hour at 37°C with slide in container saturated with water vapor.

   Catalog # P-9179
   Sigma Chemical Co.
   St. Louis, MO, USA

6. At this stage, you should be able to find the cell layers separated or at least tissue fragments from internal layer separated from the epidermal layer, permitting adequate comparisons. Longer digestion causes digestion of the tissue and thus digestion should stop after 1 hour even if tissues are not separated.
7. **Rinse** with water three times.
8. Add 1 drop of water and 3.5% **sodium hypochlorite** (household bleach) for 30-120 sec or until capsule becomes nearly transparent. (Monitor under dissecting microscope.)
9. **Rinse** with water three times for 30-60 sec each time.
10. Add **dye** for 60-120 seconds, depending on dye (see Table 1 below).
11. **Rinse** again for 60 sec in water.
12. Examine capsules in water or **glycerol**. Water can cause surface tension problems and material may scatter, making glycerol preferable (Zander 1997).
13. If necessary, gentle tapping or squashing with a pair of fine forceps may help to separate the internal layer.
14. Mountants may include **Aqueous Mountant** or **glycerol** in **glycerin jelly** (Zander 1997). Hoyer's solution is not suitable because the dye will fade.

Table 1. Von Konrat *et al*. (1999) tested coloration methods on the cell walls of the leafy liverwort ***Frullania***.

	Internal Layer		Epidermal Layer	
Stain	Cell walls	Thickening	Cell walls	Thickening
Alcian blue (0.02% w/v in water)	blue +	blue ++	blue +	blue ++
Autofluorescence	blue +	 –	blue +	 –
Bismark brown Y (1.0% w/v in 5% w/v aqueous phenol soln)	orange/brown +	orange/brown +	orange/brown +	orange/brown +
Calcofluor white (0.01% w/v in water)	blue +	 –	blue +	 –
Methylene blue (0.05% w/v in water)	blue +	blue +	blue +	blue +
p–Nitrobenzenediazonium tetrafluoroborate (0.5% w/v in 0.1 M sodium phosphate buffer pH 7.0 for 10 min at 4°C)	 –	orange +	 –	orange +
Ruthenium red (0.02% w/v in 1% w/v aqueous soln ammonium acetate)	 –	red ++	 –	red ++
Toluidine blue O (0.05% w/v on sodium benzoate buffer pH 4.4)	 –	pink–purple +	 –	pink–purple +
Nile blue A (0.01% in water)	–	–	–	–
Phloroglucinol-HCl (1 ml 2% w/v in 95% v/v aqueous ethanol + 2 ml 10M HCl	–	–	–	–
Sudan red 7B (0.1% w/v in 50% v/v polyethylene glycol + 45% v/v glycerol + 5% v/v water)	–	–	–	–

Figure 88. ***Frullania tamarisci***, in a genus that exhibits a variety of cell wall colors in various solutions (Table 1). Photo by Tim Waters, through Creative Commons.

*p*H Testing (Zander 1980; Long 1982)

Lichenologists are quite familiar with testing *p*H reactions, but this technique has not been widely used on bryophytes. Zander (1980) used *p*H responses (acid-base color reactions) to separate ***Triquetrella californica*** (Figure 89) from ***Barbula fallax* var. *reflexa*** (Figure 90) and to remove ***Bryoerythrophyllum calcareum*** and ***B. inaequalifolium*** (Figure 91) from the genus ***Barbula***. Long (1982) similarly tested four species of **Pottiaceae** and was able to distinguish them on the basis of color change. He used concentrated **HCl**, 10% **KOH**, **concentrated nitric acid**, and 2:1 concentrated $\mathbf{H_2SO_4}$, obtaining, respectively, the following results:

Bryoerythrophyllum wallichii (Figure 92) – pale brown, red-brown, red-brown, dark red-brown
Bryoerythrophyllum caledonicum (Figure 93) – pale greenish-brown, red-brown, red-brown, dark red-brown

Leptodontium flexifolium (Figure 94) – green, orange, red, brown & green
Chionoloma recurvifolium (syn. = ***Bryoerythrophyllum recurvifolium***; Figure 95) – green, orange, red, dark brownish-green

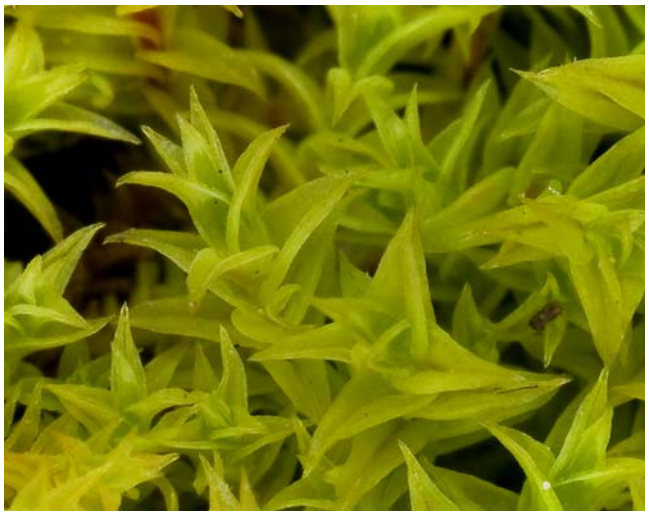


Figure 89. ***Triquetrella californica***, a species for which *p*H reactions help in identification. Photo by John Game, with permission.

Figure 90. ***Barbula fallax*** var. ***reflexa***, a species that can be separated from ***Triquetrella californica*** based on its reaction to *p*H changes. Photo by David T. Holyoak, with permission.

Figure 91. ***Bryoerythrophyllum inaequalifolium***, a species separated from ***Barbula*** by its *p*H reaction. Photo by Jonathan Sleath, with permission.

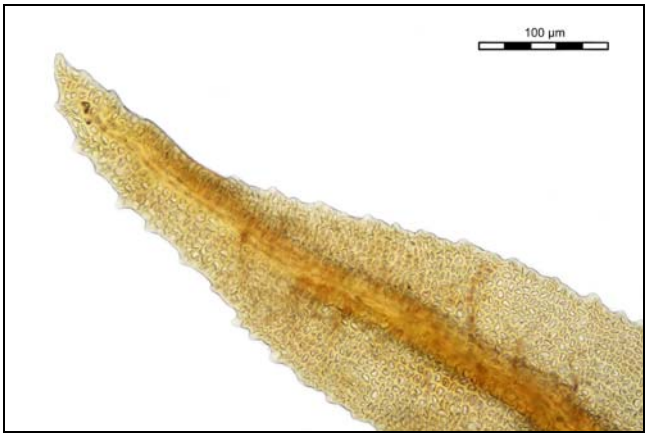


Figure 92. ***Bryoerythrophyllum wallichii*** leaf cells, a species that changes color ranging from pale brown to dark red-brown in response to decreasing *p*H. Photo from Trustees of the Natural history Museum, London, through Creative Commons.

Figure 93. ***Bryoerythrophyllum caledonicum***, a species that ranges from pale greenish brown to dark red-brown in response to changes in *p*H. Photo by Rory Hodd, with permission.

Figure 94. ***Leptodontium flexifolium***, a species that may be green, orange, red, or brown, depending on the *p*H. Photo by Dale A. Zimmerman Herbarium, Western New Mexico University, with permission.

Figure 95. ***Chionoloma recurvirostrum***, a species that exhibits colors of green, orange, red, dark brownish-green, depending on the *p*H. Photo by Michael Lüth, with permission.

Weak Alkali (Lane 1978)

Lane (1978) used a saturated solution of sodium bicarbonate (Hill 1976) in distilled water (weak alkali, final *p*H ~10) to effect color change in red-pigmented ***Sphagnum***. The branches or capitula were flooded by pipette, then permitted to stand for 1-2 minutes (Lane 1978). He then permitted the flooded branches to dry overnight, compared them to known specimens again, and flooded them with a weak acid (*e.g.* vinegar) of *p*H ~3 to check for color change reversibility. Of the 17 species tested, Lane found that there was no color change in subgenera ***Rigida***, ***Subsecunda***, or ***Cuspidata***, although ***Subsecunda*** became redder. ***Sphagnum magellanicum*** (subgenus ***Sphagnum***; Figure 96-Figure 97) became dark brown-black. ***Sphagnum wulfianum*** (subgenus ***Polyclada***; Figure 98) became chocolate brown. The nine species in subgenus ***Acutifolia*** all turned blue or dark blue. The test works equally well on fresh, freshly dried, and herbarium material.

Figure 96. ***Sphagnum magellanicum*** showing normal color variant. Photo by Michael Lüth, with permission.

Figure 97. ***Sphagnum magellanicum***, showing normal color variation compared to that in Figure 96. Photo by Jan-Peter Frahm, with permission.

Figure 98. ***Sphagnum wulfianum***, a species that turns chocolate brown at *p*H 10. Photo by Rob Routledge, through Creative Commons.

Cleaning Up Stains

Spilled stains are hard to remove. David Wagner's experience testing kitchen cleaning agents for removing stains from floors or bench tops has found "Bar Keepers Friend"™ with oxalic acid is better than most.

Leaf Removal and Making Slides

For identification, cells, margins, costa, and insertion of leaves must be seen clearly. In some cases, especially leafy liverworts, these can be seen by making a slide of the branch or stem intact. But for most mosses, it is too difficult to see everything that is needed. Removing a leaf from a moss is usually a necessity to attain this clarity. It is advisable to mount a number of leaves when leaf and cell characters need to be examined. These will represent various surfaces and positions, and greater numbers of leaves will usually provide more specimens with no interfering air bubbles.

There are a number of publications on preparing slides for viewing bryophytes (Murray 1926). I have extracted from these what works for me:

1. First **moisten the moss** by placing it in a beaker of water.
2. Place a stem on a glass slide and **strip the leaves** by pulling them downward from the tip with a pair of

microforceps while holding the tip of the branch or stem with another pair of forceps. Alternatively, you can run the convex side of a pair of curved microforceps down the stem to break off leaves. Some bryologists remove leaves by running a dissecting needle down the stem while holding the tip with forceps on a glass slide. Still others (Lucas 2009) use a spear point to run down the stem to remove leaves. Lucas points out that the spear tip tends to leave other structures such as paraphyllia on the stem where they are more easily observed.

3. **Remove most of the branches** from the portion of the stem you will observe on the slide (Lucas 2009) so that the coverslip can flatten the stem better for easier viewing. But you will also need to compare branch and stem leaves, which differ in some species.
4. Put a **drop** of water on the leaves and/or stems and spread them out so some are dorsal and others ventral in position.
5. Hold the **coverslip** by its edges and lower one side of the coverslip gently with a needle or forceps to avoid trapping air bubbles (Figure 99). If you drop the coverslip straight down, there will be no chance for bubbles to escape. If the stem is bulky and the leaves small, you might want to put them on separate slides.

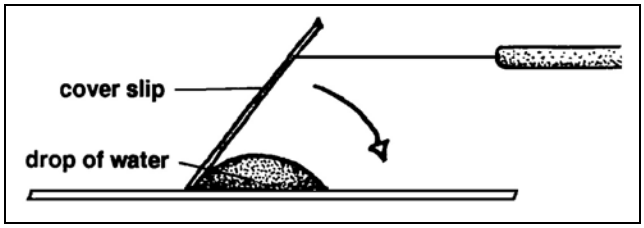


Figure 99. Technique for making a slide with minimal air bubbles. Drawing by Janice Glime.

6. If the coverslip is **floating**, remove some of the water by touching a paper towel edge to one coverslip edge. If there is **not enough water**, add water to the edge of the coverslip with a dropper. This should be added where there is adequate water at the edge to avoid trapping air as the water enters. Too much water will allow your images to move about and wiggle, making examination difficult. Too little will cause the water to draw around the specimen and cause distortions of the light.
7. Examine with the **compound microscope**. The magnification depends on the size of the specimen and what you are trying to see. It is usually best to locate the specimen and focus on 40X or 100X, then move to 400X when more detail is needed.
8. To see papillae, decurrencies, projecting costa tips, and perhaps other surface features, you need to see the leaf in side view, so it is best to observe the leaves that remain on the stem for these features. Most other features are best seen on detached leaves that are more or less flattened by the coverslip. Look around and observe several of the leaves.

Ken Kellman (pers. comm. 5 June 2015) provided me with an alternative method:

1. Etch the collection number onto a clean glass slide. I use a carbide scribe for this. I can't tell you how many times I have gotten confused about what specimen I am looking at before I started doing this.
2. First soak and dissect in a drop of water, then drop 10% glycerol onto the water drop.
3. Set that overnight to evaporate. This leaves the plants moist with most of the water gone.
4. Arrange the specimen and add a small amount of the glycerine jelly. Estimating the amount is very difficult, but you want the jelly to migrate at least to all corners of the cover slip. Too much just makes a mess. Ideally, end up with a small 5mm x 5mm x 1mm thick square.
5. Heat on a hot plate and take it off as soon as the jelly has melted.
6. Put the coverslip on and press it down to make sure the jelly goes out to the perimeter of the slip.
7. Let it cool with a weight on it. (Nuts from nuts and bolts work well).
8. After cooling, scrape the exuded jelly around the edge of the coverslip and use q-tips (cotton swabs) to wipe the perimeter of the coverslip. This has to be clean for the nail polish **lutant** (sealer) to stick. It often takes several wipes. You have to be a little careful as you don't want to keep smearing the jelly that is under the coverslip onto your slide. Keeping the q-tip wrung out helps, also continually changing the q-tip prevents smearing what is on the q-tip from previous wipes.
9. When finally clean, let the slide dry and put a coat of clear nail polish on.
10. Let that dry and put a second coat on so that the nail polish gets just over the top of the coverslip. Note that it is impossible to make this "pretty and neat." The seal is very important since it prevents (or at least drastically slows) the jelly from drying up.
11. Write a stick-on label and store it horizontally in a slide box. The etched number also helps if the label dries up and falls off, you always know what specimen the slide came from.

Avoiding Air Bubbles

Because of the small spaces among the leaves, bryophyte shoots often trap air bubbles that distort the image and make photographs less pleasing. These are hard to coax out. Sometimes it is effective to bounce the coverslip up and down with a dissecting needle or forceps.

Rod Seppelt (Bryonet September 2017) uses a mild vacuum pump attached to a water faucet to extract the air from the tissues. This is made with a Büchner funnel with a rubber bung in the top and. A Tyson of thick rubber tube is attached to the side vent of the funnel on one end and to the side vent of the faucet on the other. When the faucet is turned on, it creates a weak vacuum as it passes the side vent. The plants are put in water in the flask for this weak vacuum to remove the air. A piece of glass tubing is passed through the bung. The vacuum is controlled by the water speed through the faucet and by applying pressure with your fingers on the tube.

Anne Mills (Bryonet September 2017) reports that Bill Buck keeps a beaker full of hot water for dipping moss shoots. The added heat causes the bubbles to dissipate. The same can be accomplished by flaming a prepared slide quickly. Flaming is faster with the coverslip on, but it will sometimes break the coverslip (Dave Kofranek, Bryonet 12 April 2021).

Bubbles often get trapped in the medium when the coverslip is applied. This is especially a problem when the coverslip is dropped straight down. Most of the bubbles can be avoided by using a dissecting needle or fine forceps. With one edge of the coverslip in contact with the slide, use the needle or forceps to slowly lower one edge of the coverslip until it is entirely in contact with the water.

We need to consider a special problem with liverworts. Oil bodies in their cells are especially important in recognizing species of liverworts (David Wagner, Marc Favreau, Bryonet 12 April 2021). However, they are typically absent in dried material in the herbarium. In cases of herbarium material, wetting with hot water or using a wetting agent like Pohlstoffe or detergent that reduces surface tension is useful for combating the bubbles. The advantage of hot water is that it not only has a reduced surface tension, the heating has driven off dissolved gasses so bubbles in the mount will be dissolved. **But** all methods that use reduced surface tension for wetting are hard on liverworts. Fresh, living specimens are necessary for good photographic documentation of oil body character. These should be mounted directly in water. Stacking photography helps to visualize the oil bodies. (See Liverworts and Oil Bodies below.)

Chris Cargill (Bryonet 14 April 2021) found that heating a slide and specimen in water by using a match beneath the slide worked "perfectly" to remove air bubbles in air cavities in fresh sections of ***Ricciella***-type ***Riccia*** plants.

David Wagner (Bryonet 12 April 2021) found that best practice is to submerge the specimen in water, cover, and set aside in a small Petri dish overnight. Squirt each shoot vigorously before the plunge. The swishing helps but he thinks the overnight submersion results in the gas bubbles dissolving in the water. It is not always perfect but usually helps a lot.

Sectioning

Sectioning bryophytes is typically a hit or miss endeavor. For this reason, it is prudent to make a lot of sections (at least 10) so that at least some are likely to show what you need. If you need to see a cross section, the sections need to be thin enough for them to rest on their sides.

It seems that bryologists have developed a number of methods for sectioning bryophytes (*e.g.* Singh 1942; Foster 1977; Nishimura 1997). Nevertheless, Sean Edwards (Bryonet 30 July 2002) points out that bryologists have tended to avoid cutting sections of moss leaves for several reasons:

1. Microtome sectioning involves some considerable delay owing to the various preparations required (moreover, microtomes are often not available, especially to amateurs, when needed).
2. Pith sectioning is unsatisfactory because of the difficulty in controlling section thickness, and in separating the pith debris without damaging the sections.
3. In both microtome and pith sectioning it is almost impossible to be certain of the exact part of the leaf from which the sections were taken.
4. In both methods of sectioning, considerable care and time are needed to maintain a suitable cutting edge.

Nevertheless, there are several methods used by bryologists for making sections of stems and leaves (*e.g.* Singh 1942; Frolich 1984; Nishimura 1997). One is to place the stem with leaves on a dry glass slide and chop, like cutting parsley! The idea is that with lots of cuts, some of them will yield a usable section.

Razor Blades

Razor blades are the standard tool for cutting sections. Hutchinson (1954) recommends use of a normal (double-edged) razor blade that is divided into four sections. The blade should be placed between pieces of blotting paper and broken down the center the long way. Each of these pieces is broken again perpendicular to the previous break. She found she could use used blades because only the sharp points are needed. The blades can even be broken again when the points become dull.

Zander (2022) recommends using sharper razor blades – thin blades cut from very hard steel with a narrow bevel on each side. Triple cut bevels are more like a rounded edge and do not cut as well. Comparisons are available on the Refined Shave website <https://www.refinedshave.com/razor-blade-sharpness-summary/>. Smith (1980) claims that double-edge blades give better results than single-edge blades for free-hand sectioning, recommending those of the Feather brand.

Cutting Techniques

In the many techniques that create sections, placement of the sections is important. Once the specimen sections are in a drop of water on the slide, Hutchinson (1954) recommends stirring the water to distribute the specimens, while looking through the eyepiece of a dissecting microscope. Be sure the water is not sufficient to exceed the area of the coverslip when it is applied or you will use the smallest, hence the best, specimens. As the slide begins to dry, add 5% glycerine at the edge of the coverslip. If the best specimens need to be moved to another slide, you can use a dental applicator (Figure 100) dipped in a 5% solution of glycerine. When placed over the desired section, this combination will lift it up. The applicator can be dipped into a drop of the same solution on the new slide and the section shaken off.

Figure 100. Dental Disposable Micro-Applicators. White is superfine, yellow is fine. Photo modified from AliExpress.

Hepenstrick (2018) describes a method using a magnet to secure the slide (Figure 101). A second slide is placed across it to provide a guide for cutting (Figure 102). An instructional video is available at < https://doi.org/10.6084/m9.figshare.4781254.v1>. Beginning students who tried the magnet method agreed that it was better than the dissecting microscope with a razor blade, as seen in Figure 103.

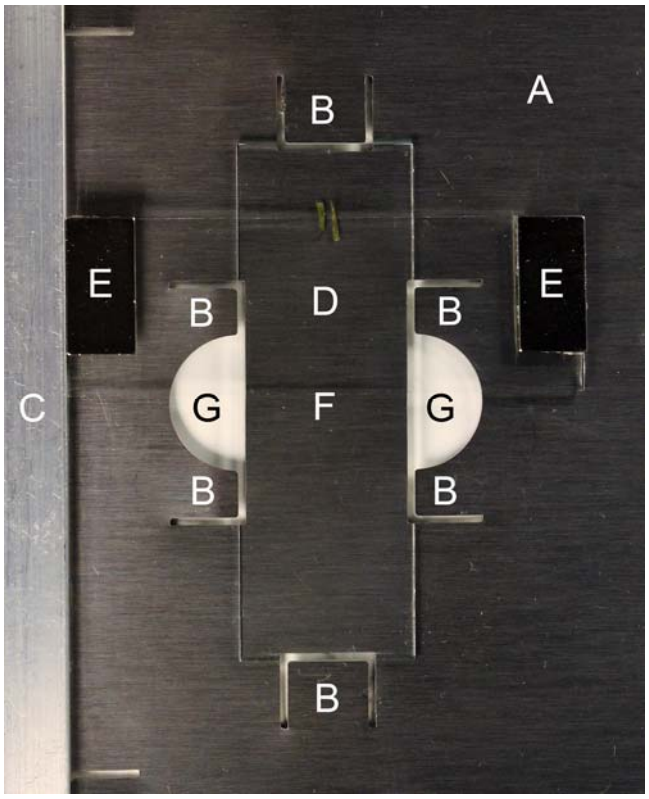


Figure 101. A magnetic sectioning aid with fixed ***Polytrichum*** leaves ready for sectioning. The support plate (**A**) is a magnetic, rust-free steel sheet with laser cut tongues (**B**) which are slightly bent upwards, and a non-magnetic strip (**C**) which helps to align the magnetic slide (**D**) with its attached neodymium magnets (**E**) centrally on the lower slide (**F**) in order to fix the specimen for sectioning. Sections can be cut under a dissecting microscope with a razor blade that is guided with both hands. The semicircle slots (**G**) help to place and grasp the lower slide. Photo courtesy of Daniel Hepenstrick.

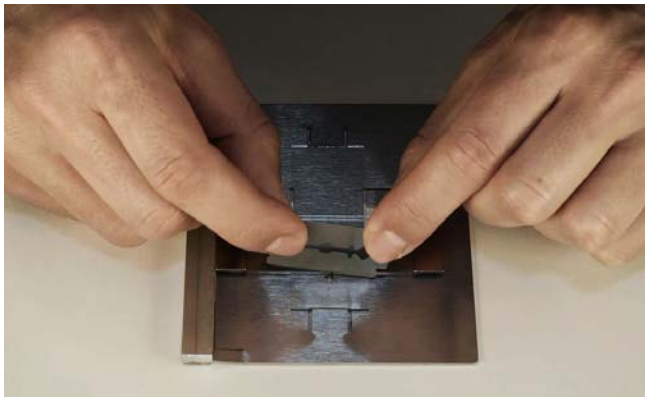


Figure 102. Sectioning with razor blade and magnetic slide holder. Photo courtesy of Daniel Hepenstrick.

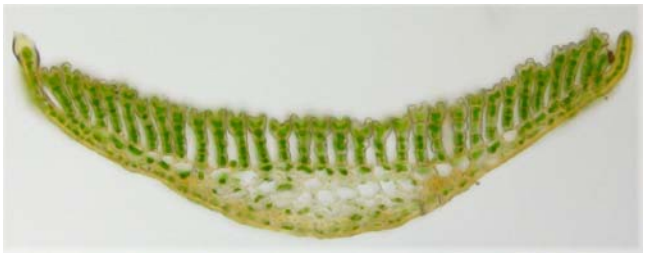


Figure 103. ***Polytrichum commune*** section cut with magnetic holder. Photo courtesy of Daniel Hepenstrick.

Wax Mounts (Taylor 1957)

Taylor (1957) found a different solution to positioning leaves and stems for cutting. He first coats them with water-soluble wax. These include Carbowax and some kinds of crayons.

Taylor makes two solutions: **Solution A** is 20% aqueous polyethylene glycol 600 with a small quantity of Quaternary amine disinfectant to prevent development of fungi in permanent mounts. **Solution B** consists of polyethylene glycols 1540 and 4000, which can be used alone or in combination. However, 1540 alone may be too soft, and 4000 too crumbly.

1. Place solution B on a slide and melt.
2. Place a piece of stem in molten drop to cover stem. The drop needs to be thick enough to support the blade during cutting.
3. Cool wax for ~1 minute with slide on cool metal surface.
4. Use quarter of razor blade to trim drop at one end to point where sectioning is to start, keeping blade vertical and at right angle to stem.
5. Keep sharp corner of cutting edge on slide with cutting edge slanting upward toward you. This keeps cutting edge sharp.
6. Move blade sideways against squared end of drop, making thinnest section possible while watching through dissecting microscope.
7. If leaves curl, soak in solution A at room temperature until solution reaches consistency of glycerin.
8. Remove leaves and touch to filter paper to remove excess liquid.
9. Transfer blotted moss to molten B and proceed from #1.
10. Transfer cut sections with adhering wax to water with small amount of wetting agent if need to keep from floating. Taylor prefers enough water to cover bottom of Syracuse watch glass.
11. Sections can be transferred by tapping slide on rim of watch glass.
12. Polyethylene glycol is not compatible with gelatin, so sections should stay in water until wax completely dissolves – a few minutes in warm water.
13. Remove sections and put in dilute glycerin onto slide.

Cutting Block (Flowers 1956)

Flowers (1956) used a 2x2x15 cm cutting block made of soft wood. She then made a jellyroll arrangement of the bryophyte in tracing paper (a thin paper):

1. Put bryophyte in boiling water to relax it and select several good, clean shoots.
2. Remove excess water by pressing the bryophyte gently between absorbent paper towels or blotters.
3. Roll a 5-10 x 30-40 mm strips of hard-surfaced, thin transparent tracing paper (such as that used by architects) lengthwise into a tight scroll. The size depends on the size of the strips. Open the roll and place the bryophyte shoots longitudinally into the first coil of the roll, using fine curved forceps.
4. Carefully roll the shoots up in the strip, using thumbs and index fingers of both hands.
5. Hold this roll up to the light to locate the upper ends of the shoots and grasp the roll just above the shoot tip with a pair of forceps.
6. Lay a strip of good quality, smooth, white cardboard (10x40 mm) parallel with the proximal edge of the cutting block.
7. Place the bryophyte roll longitudinally upon the white paper near the proximal edge, holding it down with the left index fingernail at the shoot apex.
8. Using a sharp safety razor blade, cut off the anterior portion of the paper roll and discard.
9. Begin cutting sections of stems and leaves through the tracing paper, using your fingernail as a guide. After each cut, move the blade back slightly before making the next cut.
10. As sections are cut, dip the razor blade in a drop of water on a glass slide to remove the sections.
11. Remove the sections of tracing paper from among the leaves, adding a few drops of water to facilitate the removal.
12. Excess water can be removed by holding the slide over an alcohol lamp, leaving only a thin layer.
13. Large leaves like those of ***Polytrichum*** (Figure 18) can be treated in the same way as the stems.

Pith Sandwich Cutting Tool (Trotter 1955)

1. Cut a piece of pith from common elder (*Sambucus niger*) 3-4 cm long x 1 cm wide. Make sure ends are cut clean to make a cylinder.
2. Cut cleanly as possible with sharp safety razor blade down the middle to avoid fraying.
3. Put drop of water on clean slide.
4. Lay half of pith on convex side.
5. Place **dry** specimen at end on flat side, slightly extended beyond pith.
6. Make a sandwich by placing other half of pith flat side onto the first flat side of pith, being careful to align edges.
7. Hold sandwich firmly and dip end with moss into water.
8. Place sandwich onto a glass slide without losing grip and make a first cut close to end that holds moss, using sharp, clean safety razor blade, and discard that cut.
9. Dip to wet end of sandwich again.
10. Press firmly down on the pith above the specimen and cut first section as thinly as possible **next to** the end of the pith, taking care not to cut the pith. You may want to do this while watching through a dissecting microscope.
11. After making several cuts, use razor blade or dissecting needle to move cut sections to opposite end of slide and into drop of water or wetting agent.
12. Repeat until you have enough sections.
13. Place coverslip onto cut sections and water.
14. Add extra water at edge of coverslip if needed.
15. To examine, close down the diaphragm that controls the light and examine on low power (40X total).

A modification of this method uses a razorblade instead of a slide on top (Figure 104). This is thinner and makes it easier to work with the bryophyte specimen.

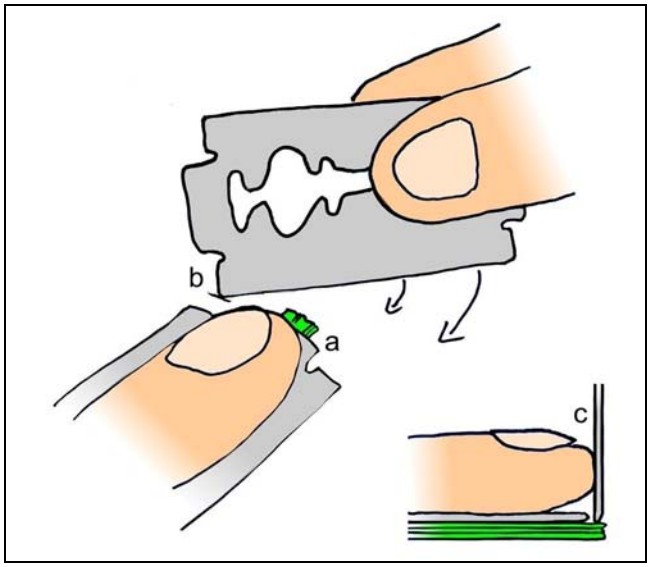


Figure 104. Lüth leaf sectioning. The bryophyte is green, shown at **a** and in the side view at **c**. The cutting position of the double-edge razor blade is at **b**. Photo courtesy of Steve Rae.

Note that sections that are visible to the naked eye are probably too thick to be useful. Note also that stems with oblique leaves, like those of ***Fissidens*** (Figure 22), may have to be placed with stems in an oblique position so that leaves are perpendicular to the cutting edge. Furthermore, plants with very brittle cells, like ***Rhabdoweisia*** (Figure 105), may make it difficult to get good sections.

Figure 105. ***Rhabdoweisia crispata***, a short species with brittle cell walls. Photo by Amelia Merced, with permission.

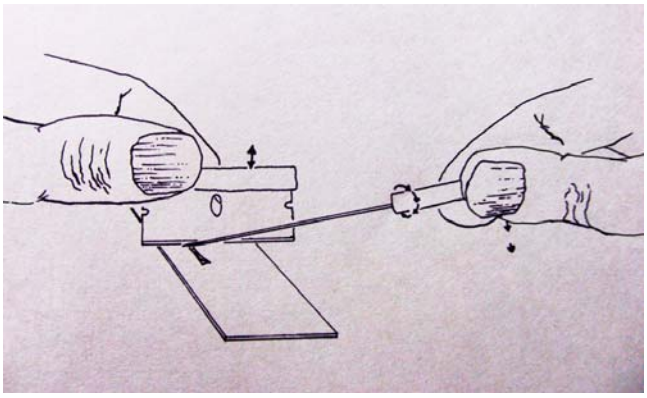


Figure 106. The roll-and-chop method of sectioning bryophytes. This would usually be done while looking through a dissecting microscope. Modified from Wilson (1990).

Chopping Method

Some bryologists subscribe to a chopping technique. They use a moist, but not flooded, stem with leaves, placed on a glass slide. These are chopped with a razor blade from the apex towards the base. Using some very fine forceps, usually adding a very small amount of water (in addition to that held between the tips by capillary action), they are spread about the water drop where the coverslip will go. After the coverslip is added, this preparation can be cleared, if necessary, by infiltrating it with a drop or two of lactic acid, and warming as discussed under Clearing Spores above. I (Glime) have always felt this chopping method was a waste of time since any chopped bits must be examined afterwards, and often none of them is useful. Most, if not all, of the sections will be wedge-shaped and won't lie on their sides. Perhaps I just gave up too soon before I perfected my skill.

Roll and Chop (Wilson 1990; Zander, Bryonet 8 July 2008)

Wilson (1990) presented a method he calls the "roll and chop" method (Figure 106). He uses a dissecting needle to hold the leaf or stem on a glass slide. After each cut, the needle is rolled back a tiny bit and cut again with the razor blade against the needle. I haven't tried this method, but I do have a concern. If one starts cutting from the bottom of the stem, the leaves become detached after the first cut, reducing the chances they will subsequently be cut in thin sections. If one starts at the tip, rolling the needle will butt into leaf tips and roll under them instead of on top of them. I asked Richard Zander for his advice on this, and he agreed that if you start at the bottom of the plant the leaves fall off. Rather, he always does "one leaf at a time if possible, since results are better. Hold the leaf down, apex away, then chop across the middle of the leaf while rolling. A substitute for rolling the needle (probe) is to hold the leaf down at an angle and slowly chop while dragging the blade down the needle; results are the same. Sometimes one can hold the whole plant down with a needle across the plant apex at an angle perpendicular to the leaves and chop across many leaves. This results in a mess but sometimes cross sections result. Less tedious than doing one leaf at a time, though."

Richard Zander (Bryonet 8 July 2008) recommends that single-edge razor blades (Figure 107) for sectioning should be discarded after five to ten uses because they become dull. He described his technique, essentially that of Wilson, on Bryonet: "One holds a leaf or stem crosswise with a stiff dissecting needle, then slices the material with a razor blade held longitudinally against the far side of the needle, meanwhile rolling the needle slowly towards oneself to gradually expose uncut portions of the material.

Figure 107. Box of single-edge razor blades. Photo by Micromark.

Practice (and a relatively fresh blade) makes this technique quite effective, even for very small leaves. Remember to scrape off sections (especially stem sections) adhering to the razor blade with a dissecting needle after cutting. The usual pair of compound and dissecting microscopes are needed, but using an additional illuminator with the dissecting microscope for fine dissections rather than just a single lamp will prove surprisingly advantageous for observation of fine features." Zander and others (Bryonet 8 July 2008) suggest Micromark <http://www.micromark.com/> as a source for razor blades.

Modified Roll and Chop (Kellman 2005)

Kellman (2005) criticized this roll and chop method because it is difficult and often produces sections that are too thick. The pressure needs to be even and sufficient to prevent the leaf from tearing. He recommended solving the first problem of thick sections by making a special needle using a sewing needle. The needle is cut to the desired length (about 7.5 cm) and inserted into a 4 cm piece of a 1 cm wooden dowel by drilling a 1.6 mm hole into the end to a depth of about 1.2 cm. The large diameter of the dowel provides one with a better grip and makes it easier to roll the needle a shorter distance. Kellman finds that the best needle is a 7.5 cm (3") soft sculpture doll needle 1 mm in diameter (Dritz product #56D). The cut end of the needle should be dipped into a drop of glue and put into the hole in the dowel. The short end of the needle can then be wedged into the hole beside the needle to position it firmly. Kellman warns that cutting the needle often results in having the cut off end flying across the room, so he recommends that it be cut inside a cloth or plastic bag so that it can be retrieved easily. The next step creates the tread that helps the needle grip the leaf. Run an emery board or sandpaper along the length of the needle, rotate the needle and repeat until the entire needle has a tread. Do not run the emery board or paper around the needle because that will not create the lengthwise treads needed.

Kellman solves the tearing and uneven pressure problem by stacking several leaves on top of each other to cut them. This also provides more sections, saving time.

1. To prepare the sections, place the stem on right-hand side of a clean slide and remove leaves under a dissecting microscope.
2. Select the leaves you want to section and move them to the left side of the slide without adding more water.
3. Once you have moved the chosen leaves, stack them together like spoons, stacking at least 3 leaves.
4. When the stack is ready, place the needle over the stack, pressing down lightly.
5. Use a sharp blade to cut along the away side of the needle. Use a chopping type of cut instead of a slice, a method not feasible with a single leaf. The full edge of the blade should reach the slide at one time.
6. Move the cut piece away and roll the needle as little as possible back toward you.
7. Make another cut, making the first section.
8. If sections get stuck to the blade, place a drop of water in the middle and dip the blade in it to remove the sections.
9. Continue this procedure until you have enough sections. Then make a slide as usual. You can place a coverslip on the stem and remaining leaves on the right to view whole leaves and another on the sections, all on one slide.

Dissecting Microscope Hand Sections (Welch 1957; Schofield 1985)

This method works well for leafy stems, branches, and large leaves. Some bryophytes, like ***Polytrichum*** (Figure 18), require leaf sectioning to view special structures like the lamellae (Figure 1). Because this is a large leaf, it is a good representative for a beginner to use for practice. Welch (1957; Schofield (1985) published the technique that works best for me (Glime):

1. Place a wet ***Polytrichum*** (Figure 18) leaf or leafy branch/stem on a dry slide.
2. Put a drop of water on one side of the slide, away from the leaf.
3. Cut away the tip with a sharp razor blade about 1/3 from the tip end of the leaf.
4. Discard your first cut.
5. While viewing through a dissecting microscope, cut as close to the previous cut as possible. Use one hand to cut and the other to guide and steady the cutting hand while holding the specimen with a fingernail or a pair of curved microforceps.
6. Cut 8-10 very thin sections and dip your razor blade in the drop of water to free them.
7. Examine the sections with the dissecting microscope to see if any of them are lying in cross section.
8. Continue cutting until you have about 30-40 sections.
9. If there are satisfactory sections, put a coverslip on the slide and examine the leaves under low and high power on the compound microscope.

With this technique I can usually get 5-8 sections (Figure 108) that will lie on their sides as they should.

Figure 108. ***Polytrichum juniperinum*** leaf cs showing several sections on a slide. Photo courtesy of John Hribljan.

Double Slide Sectioning Technique

Sean Edwards (pers. comm. 20 July 2012) has provided us with his double slide sectioning technique, based on his thesis (Edwards 1976 – see Adams 1981; Edwards 2012). The following description is only slightly modified from his description.

This method allows, with very little practice, good clean sections of about 10 µm thickness to be taken from any required part of the moss leaf, with no preparation or specialized equipment, and within a matter of seconds. The equipment required is the normal laboratory dissecting microscope (or good close eyesight), two 7.5 × 2.5 cm standard glass slides, and a supply of double-edged or single-edged razor blades. As noted by Adams in the Floating Slide Miniblade Technique, throw-away twin-bladed razors are currently in plentiful supply. By carefully prising apart the plastic mounts, two very thin, but easily hand-held ultra-sharp blades can be recovered that are ideal for section cutting (Adams 1981).

Selected moist leaves are arranged parallel with each other on a glass slide, with the parts to be sectioned aligned as shown by the arrows in Figure 109. The second slide is laid (with care) over the leaves, so that its long edge is also aligned with the parts to be sectioned (Figure 110-Figure 111). This may be checked with a dissecting microscope if necessary, and individual leaves adjusted. Firm pressure is applied to the upper slide by the finger of one hand, and half a double-edged razor blade is drawn with the other hand across the leaves, using the upper slide as a guide (Figure 110, Figure 111). Only a corner of the blade is used, but if the 'angle of elevation' of the blade is sufficiently small (about 15°-20°, perhaps less than that indicated in Figure 110), the cut is perfectly clean.

Sections are made by adjusting the tilt of the razor blade for each successive cut; the first cut is made with the blade leaning somewhat (about 15°) towards the upper slide, and this angle is progressively lessened. The situation is shown diagrammatically in Figure 112, where θ is the angle of tilt and P_1 is the fulcrum. The angle of tilt is surprisingly easy to control, and even a relatively coarse adjustment will give a fine control over the section thickness. After the tilt of the blade has passed 0° (vertical), the fulcrum moves down to P_2, resulting in an even finer control over the thickness of the last few sections. Although the sections must in theory be slightly wedge-shaped because of 15° angle, this is not noticeable in practice.

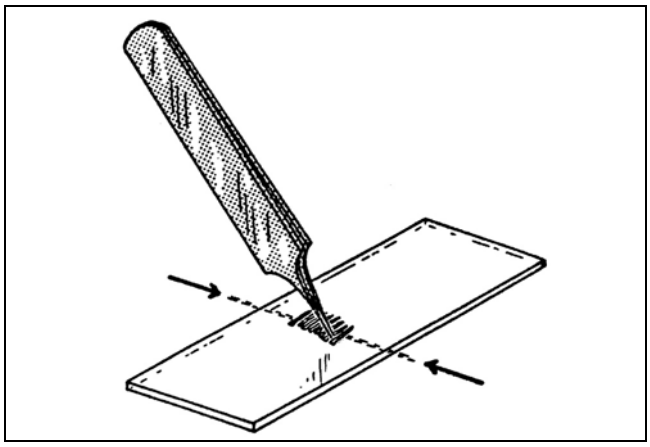


Figure 109. Placing specimen on slide in first step of the double slide sectioning technique of Sean Edwards. Drawing by Sean Edwards.

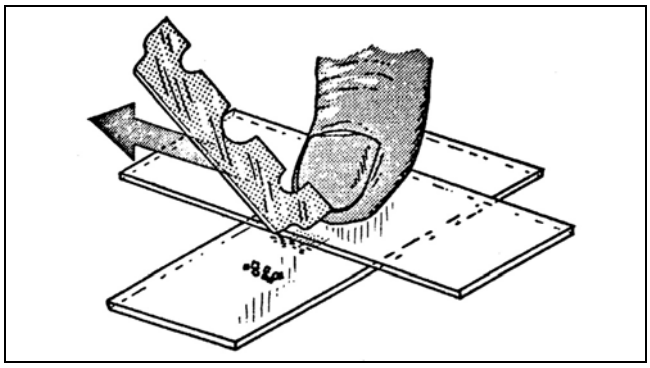


Figure 110. Alignment of slide and specimens in double slide sectioning technique of Sean Edwards. Drawing by Sean Edwards.

Figure 111. Sectioning setup of double slide sectioning technique of Sean Edwards. Photo by Sean Edwards.

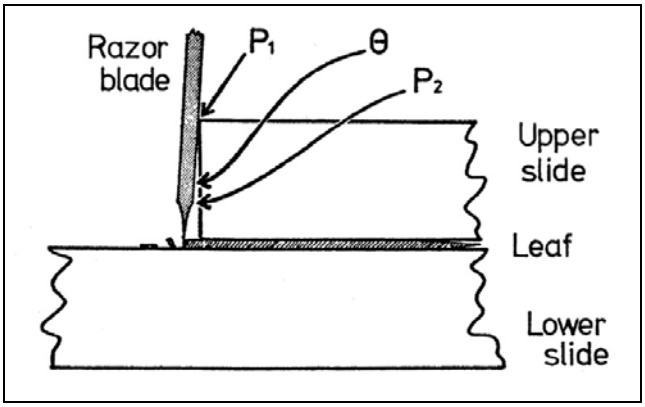


Figure 112. Cutting position of the razor blade in the double slide sectioning technique of Sean Edwards. θ is the angle of tilt and P_1 is the fulcrum. P_2 is the position of the fulcrum after the tilt of the blade has passed 0° (vertical). Drawing by Sean Edwards.

Pressure on the blade has to be judged by experience, but it should be no more than is necessary to cut the leaves. One blade corner may provide many series of sections, but such economy is usually not necessary; only with very old and fragile material should a fresh corner be used for each operation. It seems that an 'angle of elevation' of about 15°-20° enables the pressure to be taken by the less vulnerable curved corner of the blade, while allowing the razor edge unimpeded access to the leaves. It is clearly advantageous to keep this angle constant. If the broken corner of a half-blade immediately above the cutting corner is bent somewhat, just before it is first used, then the unused cutting corners can be recognized without confusion, and a packet of ten blades can be used to section at least forty plants. Particular advantages of this method lie in the degree of control and inspection allowed before and during cutting, by the transparency of the glass cutting-guide, and also in the world-wide availability and cheapness of double-edged razor blades.

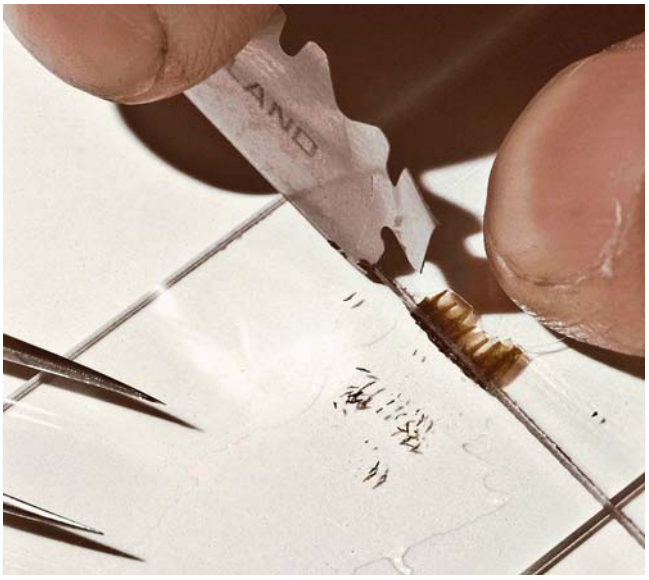


Figure 113. Cutting sections along edge of top slide in double slide sectioning technique of Sean Edwards. Note cut sections in water on the lower slide. Photo by Sean Edwards.

Figure 114. Cut sections along edge of top slide in double slide sectioning technique of Sean Edwards. Note the alignment of multiple stem pieces under top slide. Photo by Sean Edwards.

Floating Slide Miniblade Technique (Adams 1981)

This technique is nearly the same as the Double Slide Sectioning Technique of Sean Edwards, but a few modifications may be helpful to some people. The Floating Slide Miniblade Technique likewise involves two microscope slides. However, double-edged razor blades are hard to find and are really too thick to control the sectioning adequately. The second problem with the Edwards Double Slide Sectioning Technique is that the angle of tilt ensures that none of the sections are truly vertical slices with parallel slides.

Use the more readily available twin-bladed razors and pry the plastic mounts apart to obtain two very thin ultra-sharp blades. The second refinement requires the obligate use of a dissecting microscope (as also recommended by Tony Smith).

1. Mount a clean slide under the center of the low power field at ~45º to the left-right plane (near left to away right).
2. Place a large drop of water on the slide, and then mount your selection of leaves etc., in a line across the middle of the slide for sectioning.
3. Finally, add the second slide at right angles as per Edwards' technique, carefully trapping the leaves at the level you need the sections, and most importantly, a film of water between the slides.
4. Then, if you are right-handed use the second (longest) finger of your left hand (N.B. not your thumb as in Edwards' technique) to hold the upper slide down tightly onto the leaves, and using a blade in the right hand, slice away the unwanted projecting leaf segments with a gentle (so as not to blunt the blade) horizontal slice. Hold the blade vertically, but with the cutting edge tilted at ~30º towards your right hand, and slide it along the edge of the top slide, gripping the blade between the thumb and second finger, and – doing something you can't do easily with half a double-edged blade – resting your index finger along the top edge to provide that extra control.
5. Now comes the innovative bit. Because you have a film of water trapped between the slides, if you flex your left second finger slightly, you can retract the upper slide (observing all the time under the microscope) and expose new tiny projecting segments of the leaves you wish to slice.
6. Keeping your blade in the vertical plane, but with the sharp edge inclined towards your right hand at ~30º as before, you can now slice a beautifully thin section with vertical, parallel sides. With a bit of practice, it is fairly easy to retract the upper slide on its film of water, a potential slice at a time, and go on cutting ultra-thin vertical slices.

You can modify this technique by using a coverslip instead of the top slide:

1. Hold the coverglass in place with your left forefinger, with your left thumb held sideways (nail facing right), pressing down on the slide underneath. The thinness of the glass has the advantage of making it easier to see the projecting leaf segments that you are about to cut, since in the case of a slide, the edge of the glass gives you a double image. It is also easier to trap a film of water under it.
2. If you clean away unwanted bits of leaf you can lift the forward edge of the coverglass slightly with your blade, and pull it forward over the sections all in one operation, the water under it being dragged along with it and serving to suspend the sections.
3. Gripping a bunch of leaves or a shoot between thumb and forefinger, with the soft side of the thumb slightly higher, place a drop of water on the leaves and slice away against your thumb. If you are careful, you can cut a collection of slices, which stick in a film of water at the bottom of the blade, without too much damage to your thumb!
4. On dispersing the sections in a drop of water on a slide, you may find however, that they are not quite

thin enough and insist on lying flat instead of edge on. Don't despair! Pick up the best section with a pair of forceps, and mount it on the edge of a microscope slide in a film of water, as close as possible to the upper face.

5. Now despite the fact that it is lying flat on the edge of the slide, if you look at it under the microscope you will see it edge on. This is usually sufficient to enable you to observe the cell structure of a costa, and whether a margin is bistratose.

Figure 115. ***Polytrichum juniperinum* leaf section** using a cryostat. Photo by John Hribljan.

Cryostat Sections

If you are fortunate enough to have a cryostat, you can get excellent, consistent sections. I inherited a freezing cryostat that had been obtained as government surplus. The principle is that it freezes your specimen in ice. The specimen is prepared by turning the cold stage to a very cool temperature and building up an ice base with a few drops of water, waiting for each drop to freeze before adding the next. Then the specimen is placed there vertically. If you are cutting small leaves, you may want to position several of them on the ice. Once the specimen is positioned, continue to add drops of water, letting each freeze before adding the next. Once you have covered the portion of the specimen you need, you can cut off any excess with a sharp razor blade. The disk is then clamped into a holder in front of a blade. This blade (or perhaps the holder) can be moved by "winding" much like an old Victrola. Each time the blade comes down, it cuts a narrow slice from the ice and bryophyte. These must be collected on a cold, dry slide placed under the ice ribbon created – something that must be done quickly. A pair of microforceps can help to remove all the ribbon from the blade. To make the slide cold, keep it inside the cryostat while you are building the ice mound and doing the sectioning. A warm slide will melt the ribbon immediately and you can lose your slices.

The icy ribbon can be moved to the center of the slide if done quickly before it melts. Then you can add a drop of water and coverslip as you would for any slide.

The cryostat can be adjusted for the thickness of the sections. The necessary thickness depends on the thickness of the specimen (leaf, stem; see Figure 115). Capsules are a bit more difficult once they form an internal air chamber because the air will be trapped inside. If this becomes a problem, you might try adding a bit of wetting solution inside the capsule before sectioning. Be careful that the wetting agent does not get on the ice mount because the water drops will run off before they can freeze. (We haven't really tried this, so we don't know if it will work.)

Adam Hoelzer (Bryonet 5 December 2013) likewise uses a cryostat. He reports that the slices are not as thin as one can achieve with paraffin, but they are definitely adequate. Rather than using pure water, he uses the mounting medium provided by the manufacturer of the cryostat – a medium that consists mostly of polyvinyl alcohol. This is the typical medium used by hospitals that use cryostats for histology.

Stems and Small Leaves

Mosses lack lignified vascular tissue in their stems, but they may have vascular elements called **hydroids** (water-conducting elements) and **leptoids** (photosynthate-conducting elements). Additionally, the center of the stem may contain small, thick-walled cells that serve as strengthening tissue (Figure 116), but that does not seem to have any conduction function. None of these structures can be seen without sectioning the stem. Furthermore, it is difficult to section small leaves by themselves, so they are best sectioned on an intact stem or branch. This is the method that works for me (Glime):

1. Place a wet moss stem on a **dry slide**.
2. Put a **drop of water** on one side of the slide, away from the stem.
3. While viewing through a dissecting microscope, use a **sharp razor blade** to cut as close to the end of the stem as possible. Use a fingernail or finger of one hand to guide (the one holding the stem) and steady the hand holding the blade. Alternatively, you might find it easier to press down on the stem with a pair of curved forceps instead of holding it with your finger.
4. **Discard** your first cut.
5. **Cut 8-10 very thin sections** and dip your razor blade in the drop of water to free them.
6. Examine the sections with the dissecting microscope to see if any of them are **lying in cross section**, indicating they are thin enough.
7. If there are satisfactory sections, put a **coverslip** on the slide and examine the stems under low and high power on the **compound microscope**.
8. Use a microscope with **plane polarized light** to see cells with phenolic compounds in the stem.

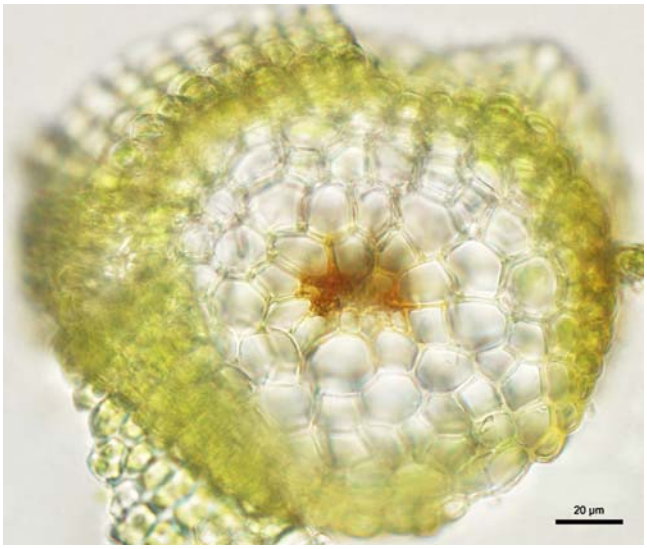


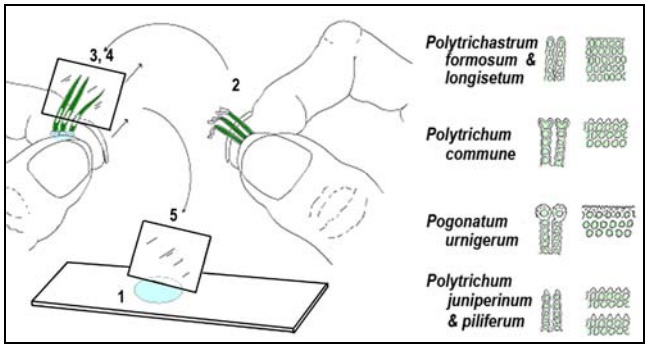
Figure 116. Stem cross section of the moss ***Molendoa sendtneriana*** showing central strand. Photo from Dale A. Zimmerman Herbarium, Western New Mexico University, with permission.

Lamellae

This sectioning technique, including the figure, is modified from the protocol by Ken Adams (2018).

When examining ***Polytrichum*** / ***Polytrichastrum*** / ***Pogonatum*** that do not have leaf margins folded over the lamellae, to determine the species, it is difficult for many people to cut very thin transverse sections. The late Peter Wanstall provided a simple solution to the problem, but beginners nevertheless seem to have difficulty with it. Hence this short procedural account:

1. Put a drop of water on a clean slide, and have some water in a dish plus a coverglass close at hand.
2. Assuming most of you are right handed, pull off a bunch of freshly wetted expanded leaves by their laminas with your right thumb and forefinger, then carefully grip the transparent sheathing leaf bases with your left thumb and forefinger so that the laminas lay across the flesh of your forefinger, before you let go with the right hand, as being springy and being bent at right angles they will otherwise fly out of line.
3. Then dip your second right finger in the water and transfer a good sized drop into the groove between your thumb and forefinger.
4. Take the coverglass in your right hand, dip the edge in this droplet, and move it away from you, gently scraping it against several laminas at once, several times, until you can see that patches of the green of the lamellae have given way to the clear tissue of the leaf lamina.
5. Dozens of lamella fragments will now be collected in a film of water along the bottom edge of your coverglass – but you are unlikely to be able to see anything – just be reassured that they are there, and dip the edge in the droplet of water you have prepared on the slide.



6. Add a cover glass and examine with the 10x objective and you should see plenty of torn-off lamellae looking like bits of green brick wall lying on their sides.

Beginners are often bewildered by these and fail to see what has happened. Imagine they really are bits of brick wall on their side. Along what was the top of the wall will be an edge with a constant repeating pattern, while along the other long edge the tissue will be torn and ragged from where it was ripped off the lamina. Students with no prior experience of microscopy may not even realize that the walls may be upside down! The top row of cells are heavily cutinized to prevent water loss, and slightly larger than the rest of the lamella cells, so that when the leaf loses turgor in dry conditions and the lamina curves slightly upwards at the edges, the top row of cells of one lamella come in contact with the top rows of lamellae on either side, thus closing off the moisture saturated air cavities in between, effectively minimising drying out of the lamellae. Using this technique the morphology of the top row cells is observed from the side, as opposed to end on as would be seen in a transverse section of the leaf. Nevertheless, because ***Polytrichum commune*** has top row cells with four large rounded bosses at their corners, in both TS and from the side two rounded bosses will be seen topping each cell, whereas in ***Polytrichastrum formosum*** and ***Polytrichastrum longisetum*** the top surface will be gently undulating in side view.

Techniques for Special Structures

Clearing Spores

Tom Blockeel (Bryonet 24 January 2012) sought a method to make it easier to see the very dark or blackish spores of species like those of ***Riccia*** (Figure 117). The ornamentation of the spores can help in identification, but it is not possible to observe it clearly with transmitted light.

Wagner (Bryonet 24 January 2012) suggested using a combination of transmitted and reflected light. The reflected light can be a strong LED light from a bicycle headlamp. This, combined with stacked images, can provide excellent quality (Figure 118).

Figure 117. Spore of ***Riccia sorocarpa*** showing its dark color and density, preventing one from seeing spore wall details without special techniques. Photo from EOL through Creative Commons.

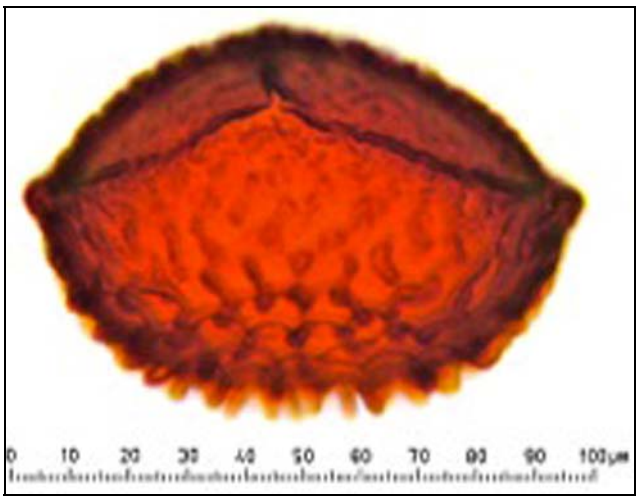


Figure 118. Spore of ***Riccia sorocarpa*** using both transmitted and reflected light plus stacking. Compare the clarity to that of the same species in Figure 117. Photo by David Wagner, with permission.

Marko Sabovljevic (Bryonet 24 January 2012) suggested using 5-10% **Clorox bleach** (NaOCl – 8% of active chlorine) for 1-3 minutes to clear the spores, a method also suggested by Richard Zander and Jörn Hentschel in the same Bryonet thread. Hentschel also suggested **calcium hypochlorite** ($Ca(ClO)_2$), the C-Solution used by lichenologists for their spot test. Martin Godfrey (Bryonet 25 January 2012) uses **gum chloral** to clear dark, dense specimens and also make a permanent preparation. But Upton (1993) reports that gum-chloral slides deteriorate steadily with time and specimens become irretrievably lost. Several bryologists (Richard Zander, Rod Seppelt, Bryonet 24 January 2012) also suggested **lactic acid**, but it wasn't clear that they had actually tried it for black spores. Seppelt also suggested a strong detergent like **Tween 80** because it reduces the black pigment in some lichens. Tom Blockeel reported that the bleach "does the trick perfectly well!" (Bryonet 6 February 2012).

An excited Richard Zander sent me an email on 15 November 2020 to share a discovery! He had been trying to clear the spores of thalloid liverworts using either 100% Clorox or lactic acid with little success. Then, he mixed the two and tried the mix on the spores of ***Ricciocarpos natans*** (Figure 119). A few bubbles arose, and "the spores decolorized and turned a very light, translucent brown, with morphologic details perfectly visible." He also used 5% Clorox and lactic acid on spores of **Targionia** (first one, then the other), and the spores again retained a brownish color. But, sadly, David Wagner tried the mix of Clorox 1:5 dilution with a drop of vinegar on spores of ***Riccia nigrella***; before he could wash them, the spores turned to mush!

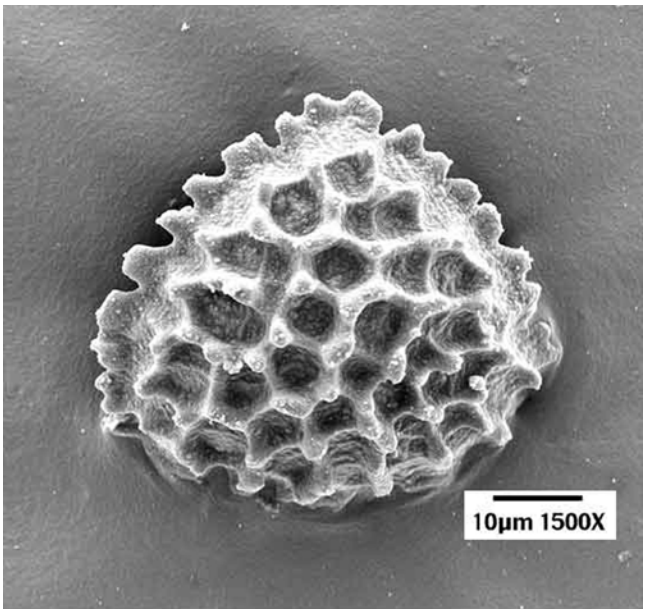


Figure 119. Distal SEM view of ***Ricciocarpos natans*** spore. Photo by William T. Doyle, with permission.

Spore Clumping and Cohesion Problems

Spores in a drop of water will tend to clump, making observations difficult. A drop of Extran® in 5 ml of water will break these apart. It might be useful to keep a bottle of water with detergent for making slides where surface tension or cohesion among leaves create problems. A little experimentation will determine appropriate amounts of detergent for various brands.

Gum Chloral Recipe for Mounting

Martin Hausler (pers. comm. 18 July 2012) provided me with this method for chloral hydrate, with the recipe originally from Watson's British Mosses and Liverworts:

Distilled water 100 ml
Gum arabic 40 g
Glycerine 20 ml
Chloral hydrate 50 g

1. Dissolve the gum arabic first in cold water, which can take a day or so as it is best not to stir to avoid getting masses of air bubbles which take an age to come out.
2. When dissolved add the glycerine and chloral hydrate and heat until dissolved; filter hot if necessary.

3. Put a drop on a slide, then add your wet specimen to it.
4. Leave the slide horizontal for a few days for the gum to set - in this time any clearing will take place. It is great for things like ***Fossombronia*** spores (Figure 120). For whole mounts of perianths or similar structures, the clearing properties will show up lots of structure without the need to dissect.

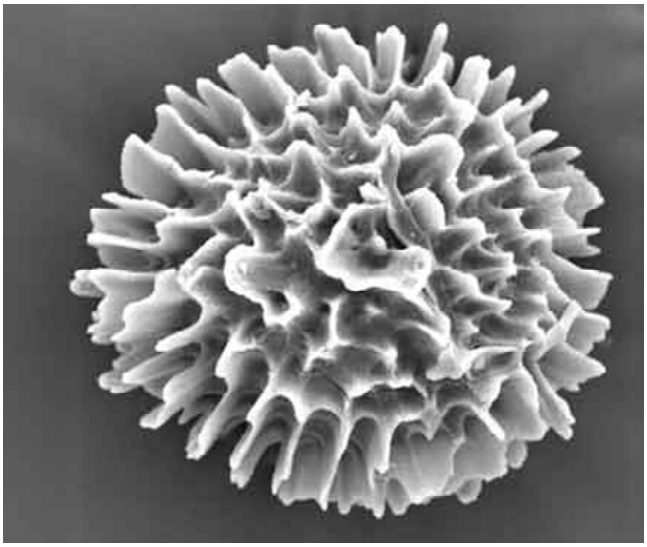


Figure 120. ***Fossombronia longiseta*** spore proximal SEM. Photo courtesy of William T. Doyle.

5. Although not strictly necessary, you may want to seal your coverslips with a couple of coats of nail varnish as it stops the gum from drying out. Technically this is a temporary mount but some slides last over 30 years old and are fine. It does tend to shrink delicate specimens so when if you don't need its clearing properties, use glycerine jelly as per Richard Zander in "Genera of the Pottiaceae."

Rod Seppelt (Bryonet 14 November 1997) suggested staining spores with **malachite green**, **acid fuchsin**, and **orange G**, a method used for testing pollen (Alexander 1969). The viable pollen stains deep red-purple, whereas the aborted pollen stains green. This recipe uses **chloral hydrate**, a controlled substance in the US. The solution uses 10 ml ethanol; 1 ml 1% malachite green in 95% ethanol; 50 ml distilled water; 25 ml glycerol; 5 gm phenol; 5 gm chloral hydrate; 5 ml 1% acid fuchsin in water; 0.5 ml 1% orange G in water; and 1-4 ml glacial acetic acid (for very thin to very thick walls). This should work as well for bryophyte spores.

SEM

Scanning Electron Microscopy (SEM) can reveal details not visible with an ordinary light microscope. Miyoshi (1969) demonstrated the intricate detail of ***Schistostega pennata*** and ***Hedwigia ciliata*** by using the Scanning Electron Microscope (SEM), compared to images using the light microscope. The image in Figure 121 was taken using SEM photography and can be compared to that of the same species using ordinary light (Figure 117) or both transmitted and reflected light (Figure 118). The SEM technique is somewhat complex and time-consuming and will not be covered at this time. Methods can be found in Hofmann *et al*. 1996, Zhang *et al*. 2007, and Srivastava *et al*. 2011, and many others.

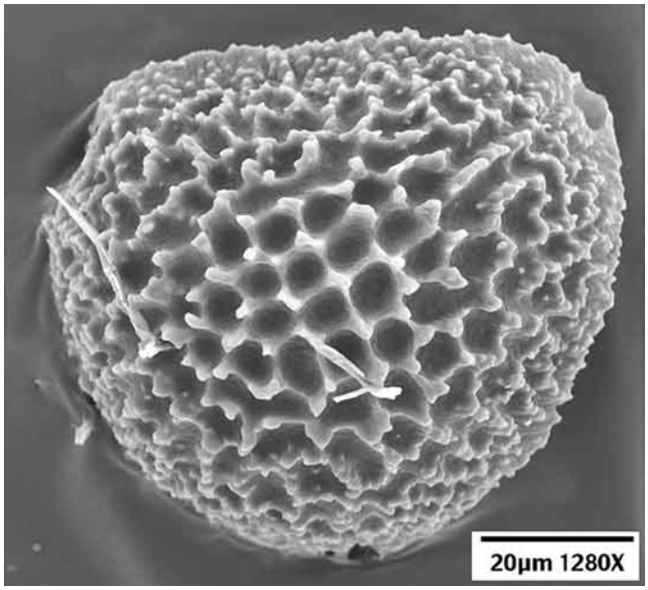


Figure 121. ***Riccia sorocarpa*** distal spore wall SEM. Photo courtesy of William T. Doyle.

Vacuoles

Many bryologists seemed to consider that bryophytes did not have vacuoles, but it appears this is only true for some taxa (Rod Seppelt, Bryonet 14 November 1997; Jeff Bates, Bryonet 14 November 1997). It is interesting that Seppelt reports that vacuoles seem to be absent in most Antarctic mosses. This suggests that absence of vacuoles may be an adaptation to cold temperatures – an interesting correlation to examine.

One indicator that a cell has a vacuole is the position of the chloroplasts (Michael Christianson, Bryonet 14 November 1997). If they are crowded around the periphery of the cell, it is likely that a vacuole is occupying the center of the cell. In Figure 122, fluorescent microscopy demonstrates the position of the chloroplasts at the periphery of the leaf cells of ***Funaria hygrometrica***, whereas in Figure 123 that is more difficult to see.

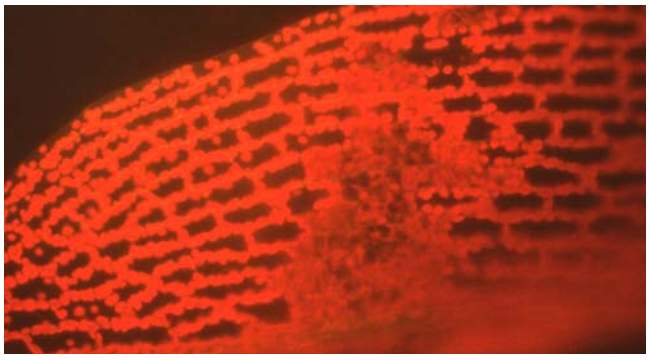


Figure 122. Leaf of ***Funaria hygrometrica*** showing chlorophyll fluorescence and demonstrating the clustering of chloroplasts at the cell margins. Such positioning indicates the presence of a vacuole. Photo by Janice Glime.

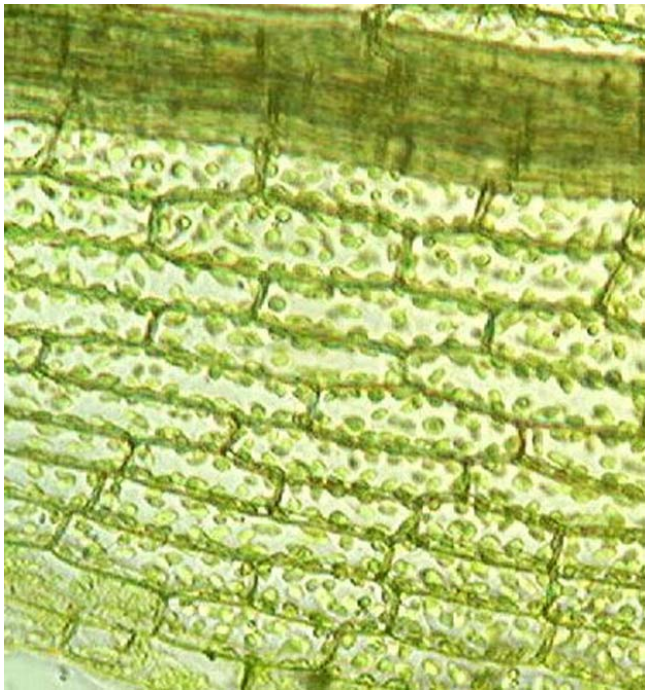


Figure 123. ***Funaria flavicans*** leaf cells showing chloroplasts on cell margins, indicating presence of a cell vacuole. Photo by Frederick B. Essig, with permission.

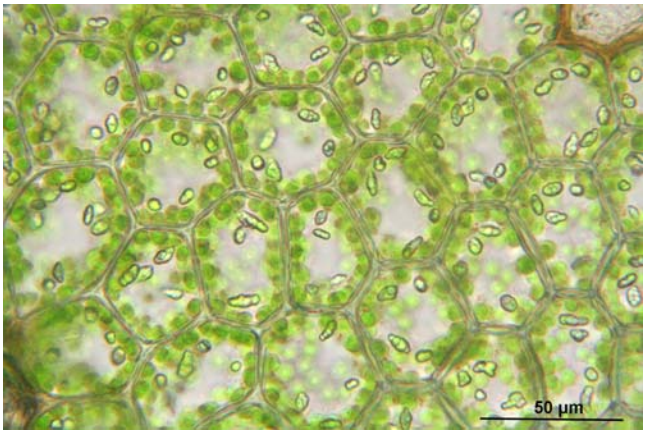


Figure 124. ***Calypogeia muelleriana*** leaf cells showing oil bodies. Photo by Hermann Schachner, through Creative Commons.

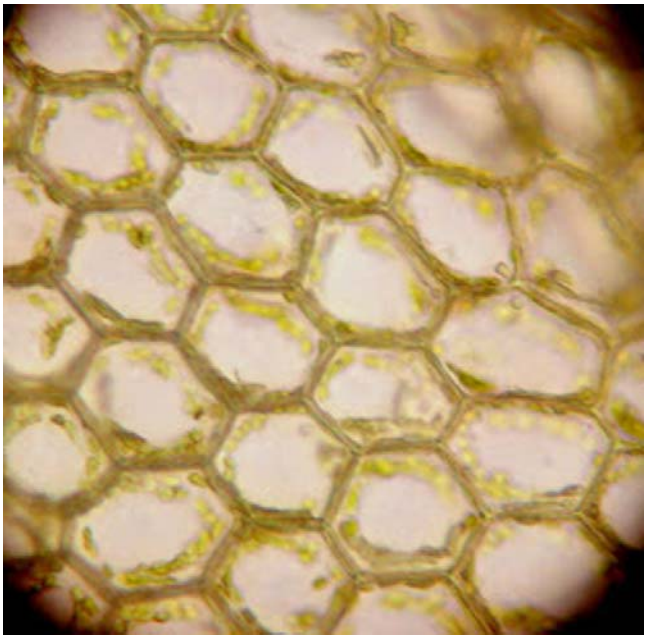


Figure 125. ***Calypogeia muelleriana*** dried leaf cells where oil bodies have disintegrated. Photo by Jutta Kapfer, with permission.

Liverworts and Oil Bodies

Oil bodies can be a problem because they disappear as the liverwort dries (Tom Thekathyil, pers comm. 27 August 2012; Wagner 2013), in some species disappearing within hours despite a moist state of hydration. David Wagner (Bryonet 5 September 2012) considers it a general rule that when cells with oil bodies die, the oil bodies dissipate. Liverworts on rotting logs (which are moisture sinks) never dry out in nature, but when they dry, they die. Unlike other bryophytes, they are not desiccation tolerant. ***Calypogeia*** (Figure 124-Figure 125) species must be examined for oil-body characters before they dry. Once dry, the oil bodies are gone forever and slow drying doesn't help. On the other hand, liverworts that grow in extreme environments, like ***Marsupella*** spp. (Figure 126) on rocks in alpine situations, are as desiccation tolerant as any bryophyte. If air dried, herbarium specimens will retain oil bodies for years because the cells are NOT dead. They live for years in a desiccated condition. To have any chance of seeing oil-bodies in dried specimens, they must be rehydrated slowly with plain water.

Sometimes Wagner has been surprised at getting good results. Also be aware that oil bodies can change character as they age after collecting. There's no substitute for immediate observation upon collections, although this period can be prolonged if they are stored in a refrigerator or cooler (Wagner 2013). On the other hand, extended periods in the dark will alter their appearance or cause them to disappear. There are some mysterious anomalies. ***Scapania gymnostomophila*** (Figure 127-Figure 128) has oil bodies that persist for decades, itself a distinctive taxonomic character.

Figure 126. ***Marsupella emarginata*** var ***pearsonii***. Alpine rock-dwelling members of this genus retain their oil bodies when they are dried. Photo by Michael Lüth, with permission.

Figure 127. ***Scapania gymnostomophila*** with gemmae. Photo by Štěpán Koval, with permission.

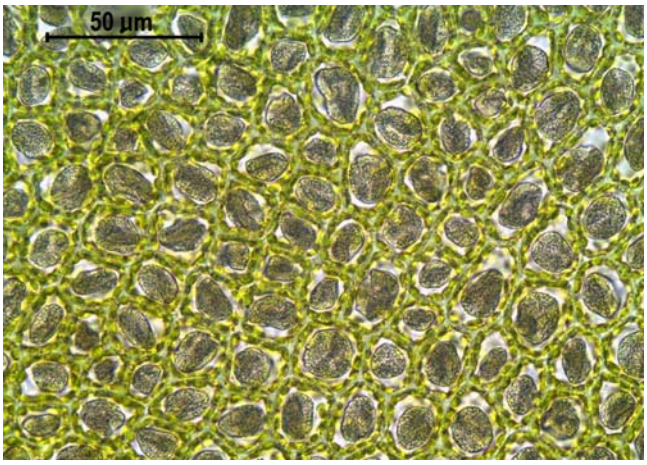


Figure 128. ***Scapania gymnostomophila*** leaf cells showing oil bodies. Photo by Hugues Tinguy, with permission.

Nevertheless, liverworts survive wetting and drying in nature. Rod Seppelt (Bryonet 27 August 2012) reported that Jeff Duckett told him that the liverworts must dry SLOWLY for the oil bodies to survive, but does this always work, or does the death of oil bodies explain why so many liverworts seem to require a moist environment?

Oil bodies are often essential for identification. Several methods of liverwort preservation have been suggested (Lehman & Schulz 1982; Ohta 1991). Lehmann and Schulz suggest a method of fixation that preserves the oil bodies, as do Müller-Stoll and Ahrens (1990). The latter researchers provide a method of staining oil bodies in live cells with diachromes and fluorochromes. If you can read the language, these may be helpful.

Peristome Teeth

Niels Klazenga (Bryonet 9 April 2014) reports the method he uses, taught to him by Dries Touw. He uses it to see teeth without having too many spores to interfere with the view. He cuts the capsule in half transversely so that you have a top half and a bottom half. Then cut the top half in four parts longitudinally. Transfer the four parts to a different slide after removing the columella and washing off excess spores. On the new, clean slide put two parts with the inside up and two parts with the outside up, so that you can see both the inside and outside of the peristome. You can do the same thing for the bottom half to see stomata, but it is rare that you need to see the inside of the bottom. These parts tend to be a bit temperamental, so they don't always end up the way you want them. Rod Seppelt added that the peristomes of some mosses assume a very different shape when wet. Instead of being straight, they bend from near the base, so that the upper parts of the teeth become strongly reflexed, completely reversing their direction. "Trying to wedge the peristome under a coverglass before wetting is tricky and does test the patience."

Brent Mishler (Bryonet 9 April 2014) suggested that one should select a recently dehisced capsule and make a longitudinal cut through the peristome (Figure 129-1&2), followed by a transverse cut slightly below the peristome insertion (Figure 129-3, 4, & 5). The endostome (attached to the spore sac) will then usually separate from the exostome with fine forceps (Figure 129-6, Figure 130-Figure 133). If a large number of spores obscure the peristomial structures, a drop of 95% ethanol added before adding water will usually disperse the spores; then excess spores can be scraped or wiped off before adding water. "Treatment with ethanol also causes the separation of the exostome and endostome, facilitating their dissection and observation. If no dehiscent capsules are available, peristome details often can be observed adequately (*i.e.* adequately for identification purposes) by removing the operculum from a nearly mature capsule by immersing the capsule in water, adding a cover slip, and boiling the slide gently over a heat source. This is usually sufficient to cause the operculum to dehisce."

To study peristomes in plane polarized light, the ventral and dorsal laminae of the teeth (not outer and inner peristomes) must be separated (Taylor 1959). Examination may even require viewing a cross section of a tooth.

1. Split capsule vertically with a razor blade.
2. Soak teeth in groups of 3-4 in 5% solution of pectinase for 24 hours.
3. Wash in 3 or more baths of distilled water.
4. Make gum syrup mountant
   A: 40 g gum arabic
      0.5 g phenol crystals
      60 cc water
   B: 52 parts cane sugar
      30 parts water (by volume)
   Combine 25 cc A, 15 cc B, and 2 g glycerin.
5. Cover a small area of a slide with a thin coating of the gum syrup mountant.
6. For peristomes, permit gum arabic to become almost dry.
7. Transfer teeth in groups of 3-4 to mountant, making sure some groups show the ventral and others dorsal surface.
8. If peristome teeth curl, they can be moistened slightly with a damp (not wet) fine water color brush (# 00000).

9. The gum arabic can be remoistened if needed, especially if used for leaves and other structures.
10. The teeth can be flattened on the slide with a needle or the damp brush.
11. Make sure the gum syrup is nearly hard, but soft enough to flow under pressure. This will take practice to prevent ripples from too much liquid, but must permit the teeth to pull apart.
12. To make teeth very flat (desirable), cover part of slide lightly with light coating of paraffin wax or other substance to prevent the adhesive from sticking to it.
13. Press the coated slide against the teeth until they are tightly pressed against the mountant.
14. Permit the gum arabic mountant to harden.
15. Remove uppermost surface of lamina on each set of teeth by gentle scraping, using a dull tool such as a discarded side-cutting dental tool.
16. Remove the loosened particles with a dry brush.
17. Lightly moisten the gum syrup to get a smooth surface and allow to dry.
18. To make the slide permanent, add the desired finisher, such as gum-chloral.

To View Teeth:

19. Place the finished slide on the rotating stage of a polarizing microscope and turn stage to a position where light is extinguished when viewing slide.
20. Insert gypsum tube into microscope tube and rotate stage clockwise.
21. If tooth lamina becomes blue or green, chains run N-S when tooth is returned to this extinction position.
22. If tooth lamina becomes yellow after rotation, search for a position at right angles and repeat the test.
23. Be careful not to rotate counter-clockwise.
24. If all chains are parallel, you will not find the bright color change, but usually at least some will show an acute angle between two sets of chains.

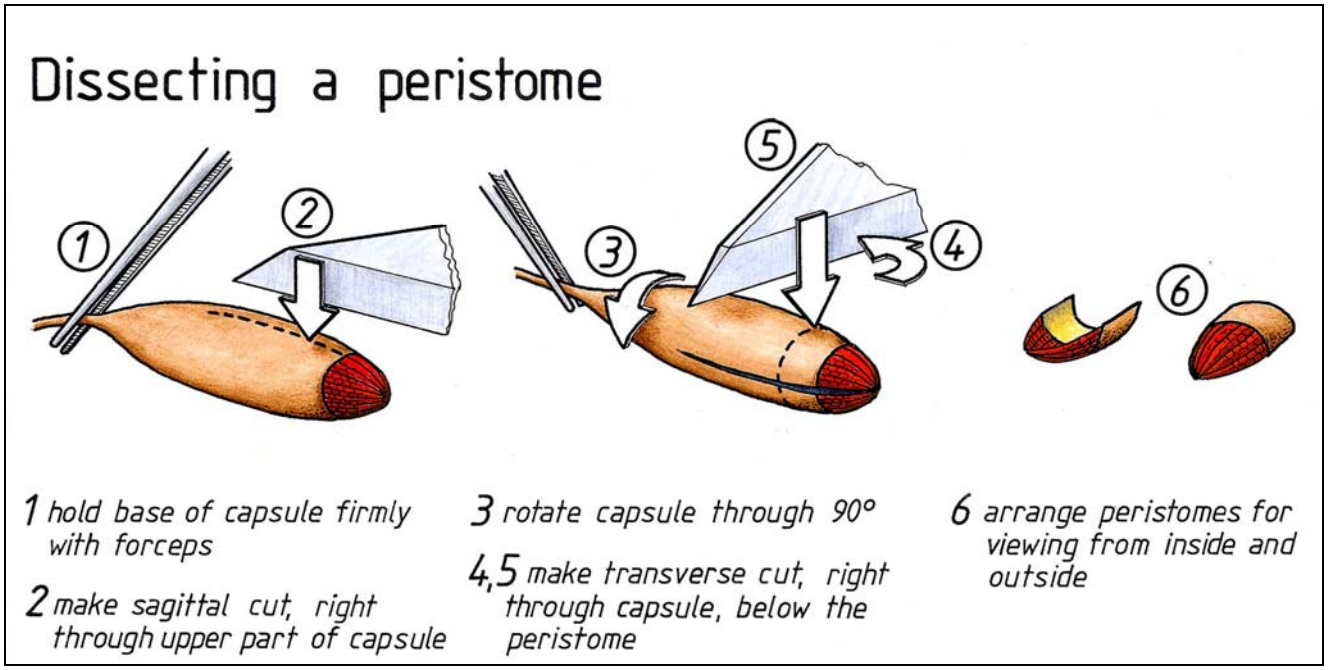


Figure 129. Cutting method for capsule to display peristome teeth on a slide. Drawings by Sean Edwards.

Javier Penalosa (Bryonet 12 April 2014) found that slightly boiling the slide makes the operculum pop off the capsule (see Figure 130, Figure 132). He was successful in using this technique to see peristomes of ***Bryum*** (Figure 130, Figure 132) and ***Brachythecium*** (Figure 133). Once the operculum is off, a drop of alcohol will disperse spores on the slide. This exposes the **nodose cilia** in ***Brachythecium rutabulum*** (Figure 133) and appendiculate cilia in ***Brachythecium oedipodium***.

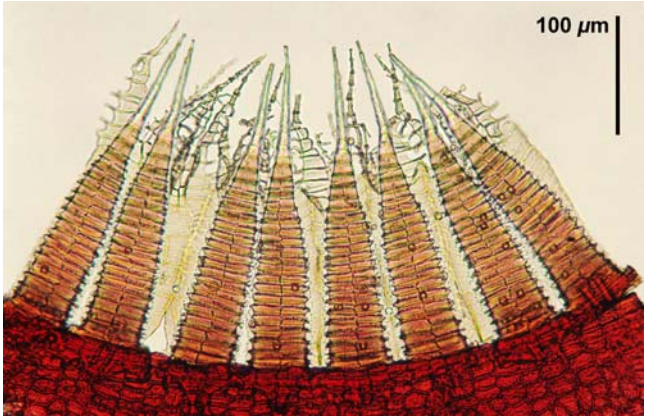


Figure 130. Peristome teeth of ***Bryum dichotomum***. Photo by Sean Edwards.

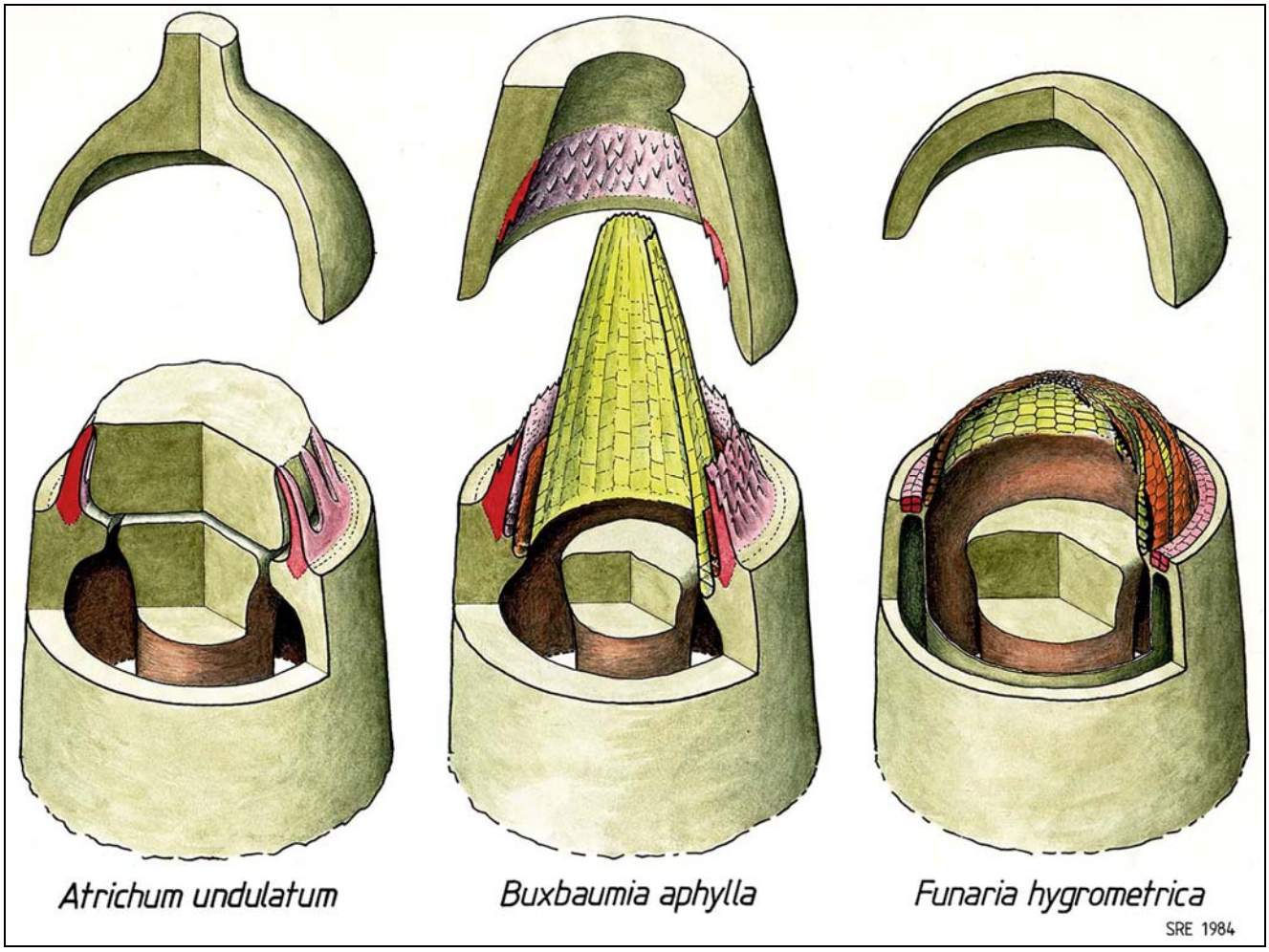


Figure 131. Three species with the operculum removed to reveal differences in peristome types. Drawings by Sean Edwards.

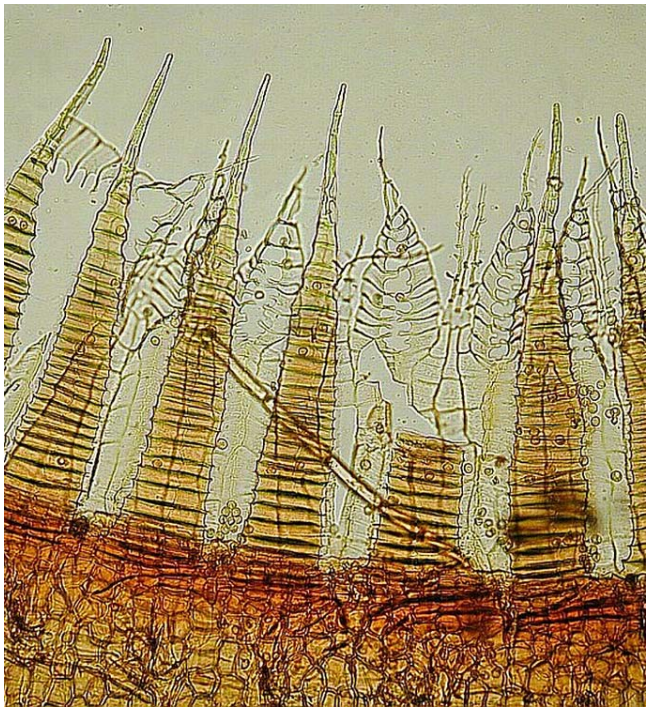


Figure 132. ***Bryum caespiticium*** peristome showing peristome teeth and cilia. Photo by Michael Lüth, with permission.

Figure 133. ***Brachythecium rutabulum*** peristome showing nodose cilia (see sever in upper part of image between inner and outer peristome). Photo by Laurie Knight <www.laurieknight.net>, with permission.

Summary

Bryophytes often need to be cleaned before they are mounted for observation. Methods for doing this include a special bryophyte washing machine, netting on an embroidery hoop, wash bottle, HCl, H_2O_2, and agitation. Dried bryophytes need to be rehydrated using a wetting agent such as water, soap, detergent, heated water, 2% KOH, Pohlstoffe (docusate sodium), or Agral 600. Some leaves need to be cleared before cell wall papillae and wall structure can be seen clearly, using reagents such as lactic acid, KOH, NaOH, or chloral hydrate. Some species require air drying or dehydration in ETOH to prepare them for making a slide.

Stains permit further clarification of structures such as pores and wall markings and permit determination of cell types. They can be as simple as food coloring or an array of chemical stains used singly or in combination. Identification of ***Sphagnum*** usually requires a stain to discern the leaf cell pores. Archegonia and spermatogenous cells can be stained with fast green. Fluorescent dyes coupled with a fluorescence microscope can reveal bulbils and determine if spores are viable. A pectinase preparation can be used to stain liverwort capsules.

Some bryophytes (esp. Pottiaceae) produce different colors in reaction to a mix of HCl, KOH, concentrated H_2NO_3, and H_2SO_4. Some ***Sphagnum*** subgenera respond to *p*H and have distinctive colors in $NaHCO_3$.

Removing leaves from stems is aided by a dissecting microscope and microforceps. Sharp razor blades can be used to make sections of leaves and stems. Cutting is best done under a dissecting microscope, with the method being largely a matter of preference, including chopping, wax mounts, pith sandwich, cutting block, and double slide sectioning. If you are lucky enough to have a cryostat, you can use it to make sections.

Some structures require special treatment, such as clearing spores, using SEM, seeing vacuoles, preserving and seeing oil bodies, and seeing details of peristome teeth.

Acknowledgments

Numerous discussions by Bryonetters have contributed heavily to this chapter. I appreciate the additional help from Richard Zander who answered my many questions quickly and thoroughly and alerted me to his websites. He also did further testing of the clearing technique. Isawo Kawai kindly sent me numerous pictures and information on his staining procedure. Ralf Wagner provided me with the instructions for the Etzold staining and W3A staining.

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CHAPTER 2-2b
SPHAGNUM STAINING

H. Rudi Zielman and Janice M. Glime

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CHAPTER 2-2b
SPHAGNUM STAINING

Figure 1. ***Sphagnum russowii***, a species for which pores are seen more easily when stained. Photo by Des Callaghan, with permission.

Following a discussion on Bryonet and the diminishing size of his trusty aniline pencil (Figure 2), Rudi Zielman set out to compare various stains used to make the pores of ***Sphagnum*** leaves and stems more visible. This subchapter is the result of that investigation. Another driver for this investigation is the toxicity of aniline. Furthermore, newer versions of this pencil simply didn't work – they didn't color wet leaves (Figure 3-Figure 4). And an aniline solution did not color the leaves easily. Then the leaves lost their color when they were placed in water.

Figure 2. Aniline blue pencil used to stain ***Sphagnum***. Photo by Rudi Zielman.

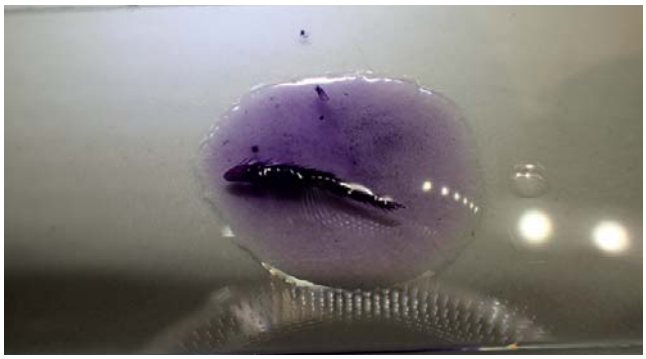

Figure 3. ***Sphagnum obtusum*** branch in water with aniline blue pencil scrapings. Photo by Rudi Zielman.

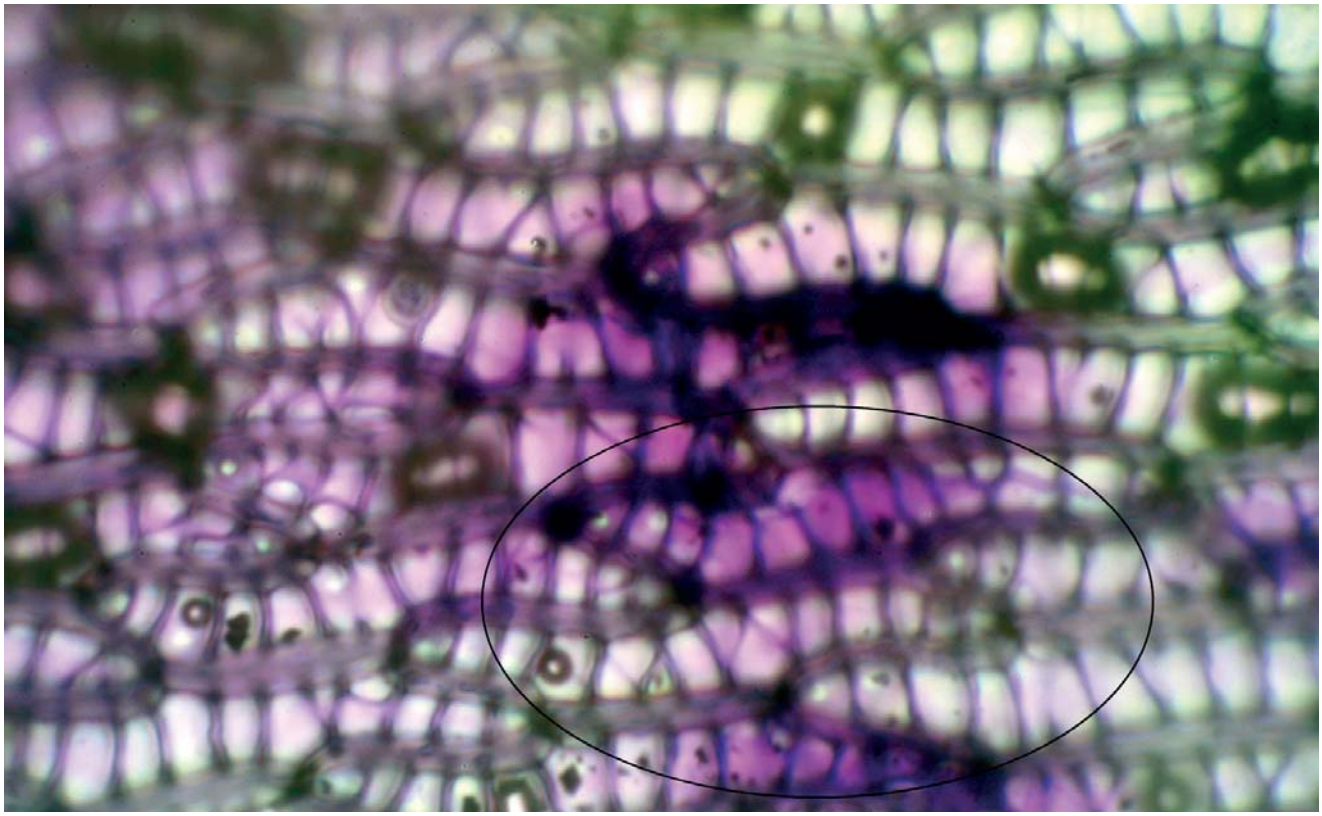

Figure 4. ***Sphagnum obtusum*** stained with an old aniline pencil. The branch was stained, leaves carefully removed, and placed on a slide in water. The pores became more visible, as seen in the area inside the ellipse. Photo by Rudi Zielman.

The dyes used are all dyes with a ring structure; especially with toluidine blue it is emphasized that it should be the toluidine blue-O, *i.e*. the methyl and amine groups in the ortho position. This is also true with safranin, and apparently is the case in all the stains described here and currently available for staining the moss. Methylene blue and safranin are sold at (web) stores that also sell microscopy equipment.

Toluidine blue is currently the most difficult to obtain; when Zielman collected all the materials about 8 years ago he was able to collect the needed materials from a web shop and a university lab; gentian violet was also a problem at that time, now to a lesser extent; one needs a doctor's prescription to get it from a pharmacist. It is used in the treatment of **thrush** (a fungal infection) in infants, and can apparently also be found at shops that focus on supplies for breastfeeding. An advantage is that all these dyes are, in contrast to aniline, non-toxic. A 10 ml ready-to-use solution costs approximately 15-20 euros. Methylene blue, toluidine blue-O, and gentian violet are also available as a powder (quite difficult); to use them one places a few grains (forceps tip) on a slide and dissolves this in water or ethanol. You can also use the powder to prepare a "stock solution" (additional recipes on the internet; several are listed here, but unfortunately no URL's or author names were available), but then some stirring and filtering facilities are required. For the staining effects it does not matter whether you use the solution or the powder, but the solution works more easily.

A word about safety: methylene blue is the most annoying of these four dyes tested here. It is non-toxic, but it can cause eye and skin irritations. All solutions contain alcohol and are therefore slightly irritating. Spilled dyes can be easily removed with a tissue and some methylated alcohol.

Methods

The stains used are:

No colorant (stain) applied
Methylene Blue
Toluidine Blue (actually tolonium chloride)
Gentian Violet (also called crystal violet or methylrosanaline)
Safranin

Methylene Blue from Powder

Prepare a saturated solution of methylene blue by adding 1.5 g powdered methylene blue to 100 mL 95% ethyl alcohol. Slowly add the alcohol to dissolve the powder. Add 30 mL saturated alcoholic solution of methylene blue to 100 mL distilled water and 0.1 mL 10% potassium hydroxide. Always make these in a 1% ETOH solution, a saturated solution in water.

Toluidine Blue-O from Powder

Dissolve the toluidine blue powder in distilled water (0.1 g of toluidine blue in 100 ml of distilled water). Check the *p*H of the solution, it is very important. The stock solution should be *p*H 2.3 (and less than 2.5), achieved with 5 ml 1% sodium chloride in 45 ml; mix well. The working solution should be *p*H 2.0-2.5. Make this solution fresh and discard after use.

Alternatively, mix powder to dissolve and adjust *p*H to 2.0~2.5 using glacial acetic acid or HCl.

Gentian Violet Powder

Dissolve 2 g of gentian violet powder dye in 20 ml of 95% ethanol (Histanol 95) and mix with 80 ml of 1% aqueous solution of ammonium oxalate. Dip the moss quickly and rinse in water (Figure 5).

Figure 5. ***Sphagnum*** stained with gentian violet. Photo by Don Avery, with permission.

Safranin-O

Mix 10 ml of basic solution with 90 ml of distilled/demineralized water.

Applying Stain

For each stain, a dry branch or stem piece is quickly dipped in a few drops of the stain, stirred and slightly pressed to make sure the stain is distributed everywhere. If you dip too briefly, the leaf parts (often the proximal half) may not be properly stained because the stain solution could not reach them. After dipping, the branches or stem pieces are rinsed in demineralized water. Do this carefully; ***Sphagnum*** mosses very easily lose leaves or become damaged. Just dip in water, replace drops, re-dip, until the water no longer colors. After that, the material is mounted on the slide.

Microscopic images in this subchapter were taken with a Leica DM E microscope with 40 X achromatic objective and trinocular head with a Leica 1 X photo lens on which a Nikon D5300 camera body was attached. The diaphragm opening of the microscope is equal for all photos; the exposure intensity is not. Because a microscopic image has no depth of field, stacking is needed. First focusing is done slightly above the leaf blade or section and then the fine adjustment knob is used manually through small steps to change the focal plane through the cell wall, going deeper and deeper. The recordings are then stacked with CombineZM and reworked (color levels automatically balanced, stack edges clipped) with GIMP 2.10. (It is also possible to have a camera that does automatic stacking and combining the images.) The resulting photos are composed of a variable number of individual photos, depending on visual evaluation (or the number provided by an automatic camera).

Results

In the overviews below we show a few species in which pores are important to observe. For each species in the images shown, the different stains were applied to adjacent branches of the same stem just below the capitulum. The order is always no coloring, methylene blue, toluidine blue-O, gentian violet, safranin. This sequence shows a fairly even gradient in the colors seen, from blue through purples to orange-red.

Sphagnum divinum (Figure 6-Figure 16)

First of all, ***Sphagnum divinum***, where the width of pores in the proximal part of branch leaves and the thickness of the wall between **chlorocytes** (cells with chloroplasts) and **hyalocytes** (colorless cells) are important to observe. What you see in these images of ***Sphagnum divinum*** is that the pores in the hyalocytes are clearly visible and are less than half the width of the cell. The leaf cross section is less clear. This is caused by the sigmoid cell pattern; the wall between hyalocytes and chlorocytes is visible through many sections behind one another, and thus is often blurred in stained leaf transections, so it is recommended to inspect these in unstained condition.

Figure 6. ***Sphagnum divinum***, Ireland, a segregant from ***Sphagnum magellanicum*** that can be identified more easily when stained. Photo by David Long, with permission.

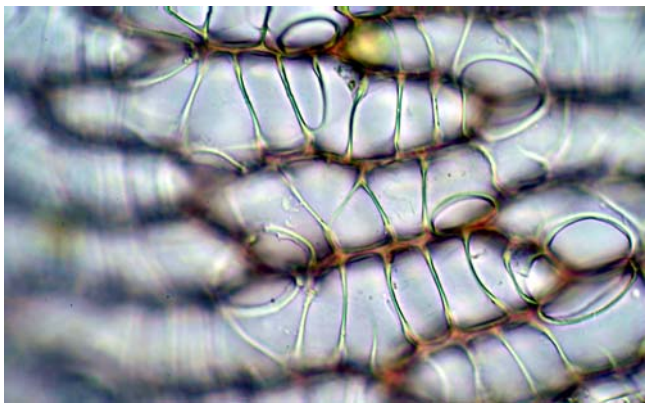

Figure 7. ***Sphagnum divinum*** leaf cells with no staining. Photo by Rudi Zielman.

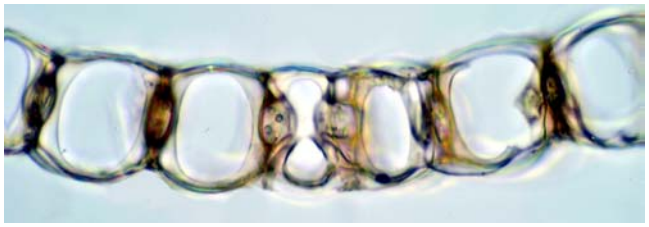

Figure 8. ***Sphagnum divinum*** leaf cross section with no staining. Photo by Rudi Zielman.

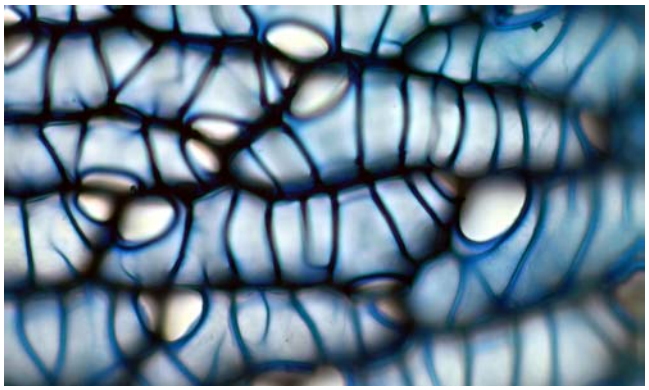

Figure 9. ***Sphagnum divinum*** leaf cells stained with methylene blue. Photo by Rudi Zielman.

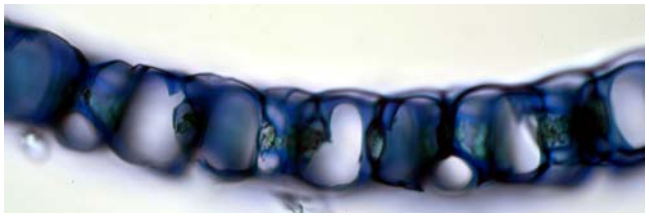

Figure 10. ***Sphagnum divinum*** leaf cross section, stained with methylene blue. Photo by Rudi Zielman.

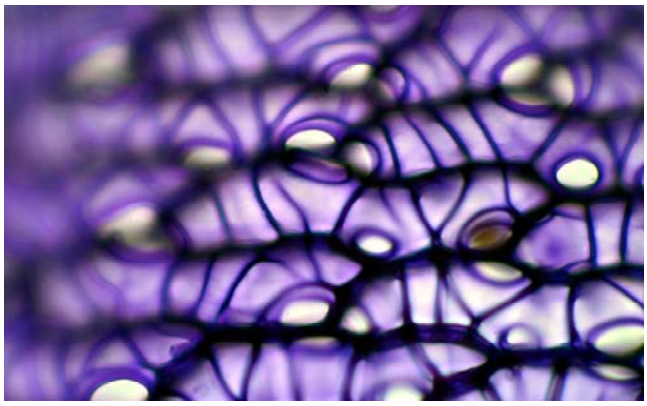

Figure 11. ***Sphagnum divinum*** leaf cells stained with toluidine blue-O. Photo by Rudi Zielman.

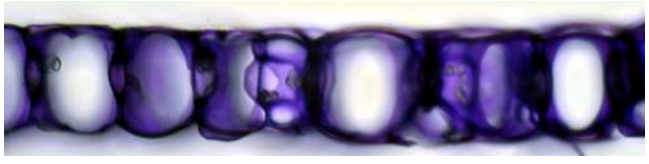

Figure 12. ***Sphagnum divinum*** leaf cross section, stained with toluidine blue-O. Photo by Rudi Zielman.

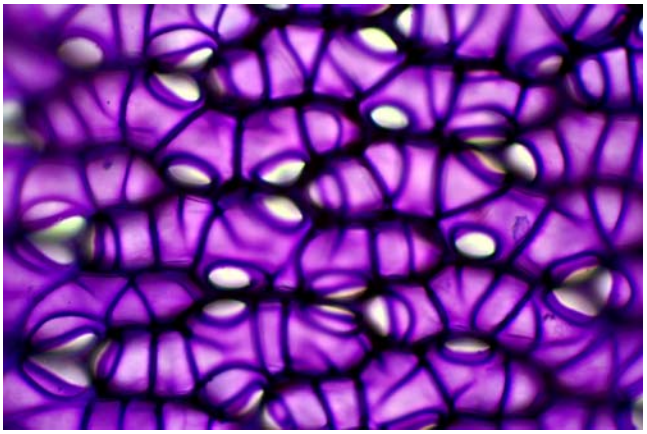

Figure 13. ***Sphagnum divinum*** leaf cells stained with gentian violet. Photo by Rudi Zielman.

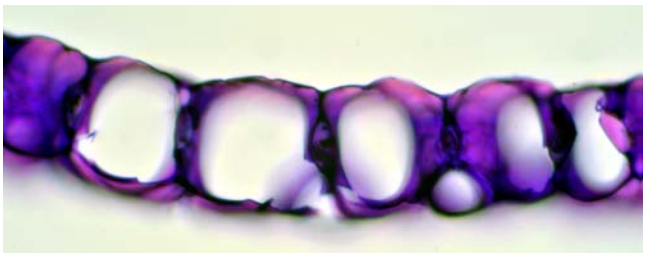

Figure 14. ***Sphagnum divinum*** leaf cross section, stained with gentian violet. Photo by Rudi Zielman.

Figure 15. ***Sphagnum divinum*** leaf stained with safranin. Photo by Rudi Zielman.

Figure 16. ***Sphagnum divinum*** leaf cross section, stained with safranin. Photo by Rudi Zielman.

Sphagnum obtusum (Figure 4, Figure 17-Figure 27)

A true challenge with staining lies in making visible the very small and very unclear pores of ***Sphagnum***

obtusum. The cell wall thinnings that matter most are primarily located proximally in the leaf at the lateral sides; this zone is therefore always pictured.

Figure 17. ***Sphagnum obtusum***, a species with faint pores that require staining for observation. Photo by Michael Lüth, with permission.

It should be clear that all stains enhance the visibility of the structures in the branch leaf cells of ***Sphagnum obtusum***, while without such staining the faint pores remain invisible. But again, the stained cross-sections of the branch leaves are more difficult to interpret than the unstained ones.

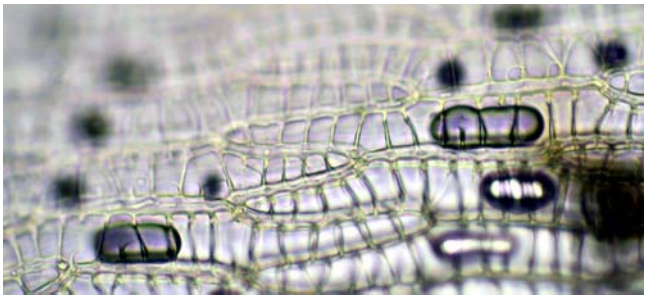

Figure 18. ***Sphagnum obtusum*** leaf cells, with no staining. Photo by Rudi Zielman.

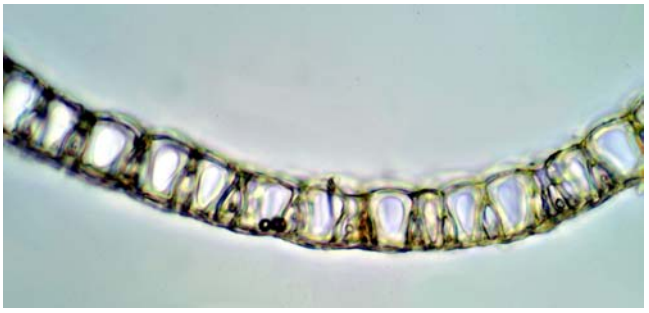

Figure 19. ***Sphagnum obtusum*** leaf cross section, with no staining. Photo by Rudi Zielman.

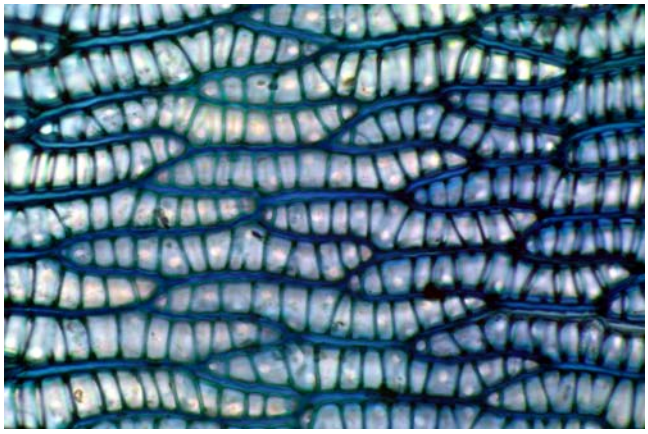

Figure 20. ***Sphagnum obtusum*** leaf cells, stained with methylene blue. Photo by Rudi Zielman.

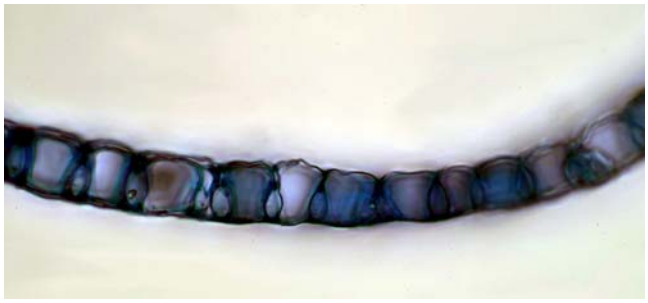

Figure 21. ***Sphagnum obtusum*** leaf cross section, stained with methylene blue. Photo by Rudi Zielman.

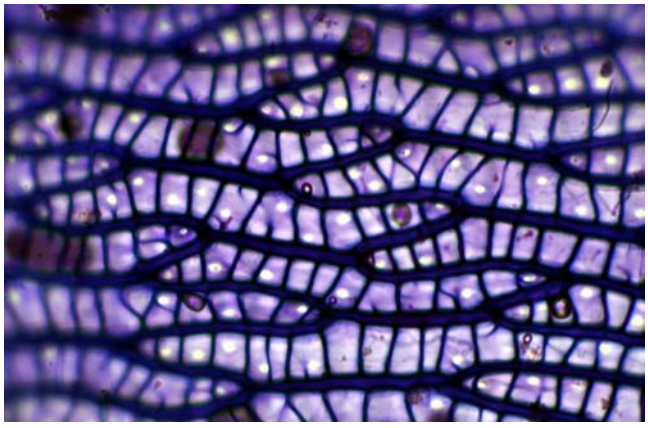

Figure 22. ***Sphagnum obtusum*** leaf ells, stained with toluidine blue-O. Photo by Rudi Zielman.

Figure 23. ***Sphagnum obtusum*** leaf cross section, stained with toluidine blue-O. Photo by Rudi Zielman.

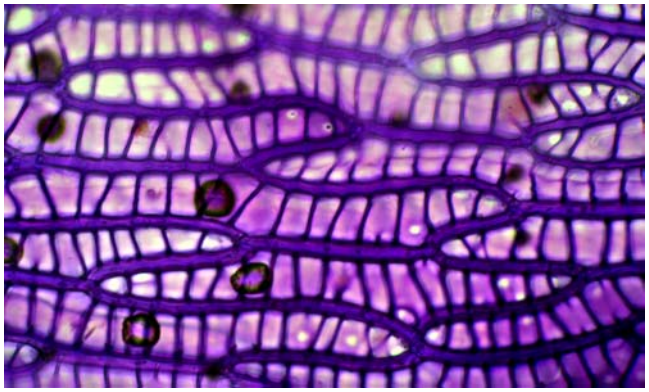

Figure 24. ***Sphagnum obtusum*** leaf cells, stained with gentian violet. Photo by Rudi Zielman.

Figure 25. ***Sphagnum obtusum*** leaf cross section, stained with gentian violet. Photo by Rudi Zielman.

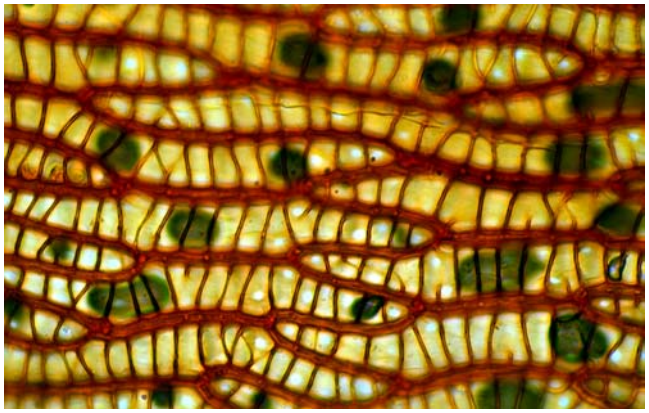

Figure 26. ***Sphagnum obtusum*** leaf cells, stained with safranin. Photo by Rudi Zielman.

Figure 27. ***Sphagnum obtusum*** leaf cross section, stained with safranin. Photo by Rudi Zielman.

Sphagnum russowii (Figure 28-Figure 38)

In ***Sphagnum russowii***, the **pseudopores** (thin spots in the cell wall) of the stem epidermis are of importance. The easiest way to prepare them is by holding a piece of stem with forceps and then cut the whole stem diagonally with a razor blade; sometimes it even works to get rid of the tissue below that epidermis completely (*e.g.* in the gentian violet preparation in Figure 36). Hölzer (2010) also mentions the large pores on the ventral side of branch leaves as characteristic (Figure 29); figs 30, 32, 34, 36 show the same pore structure.

Figure 28. ***Sphagnum russowii***, a species with pores that are more easily seen with staining. Photo by Hermann Schachner, through Creative Commons.

Figure 29 is the non-stained version of ***Sphagnum russowii*** leaf pores; this image comes close to what we see through the microscope. In all pictures of the stem epidermis (Figure 32, Figure 34, Figure 36, Figure 38), except the unstained (Figure 30), the faint pores are clearly visible. Also the large pores on the ventral side in the branch leaves are easily recognizable. Please realize that the white holes are a view where a pore on the ventral and dorsal side are aligned with each other!

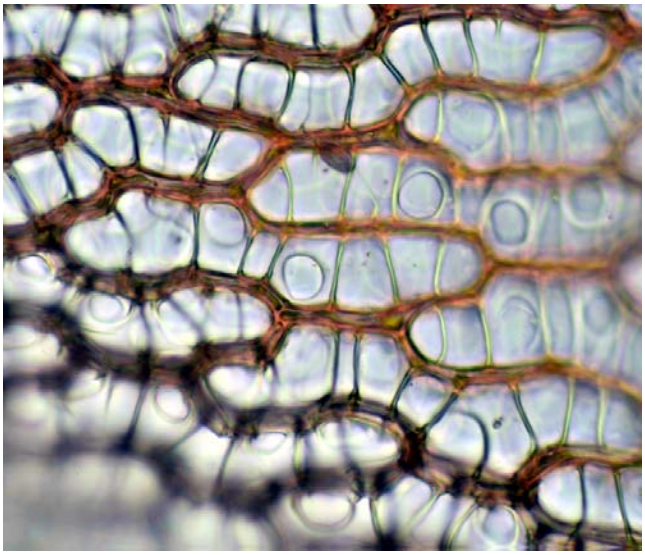

Figure 29. ***Sphagnum russowii*** leaf cells showing pores with no stain. Photo by Rudi Zielman.

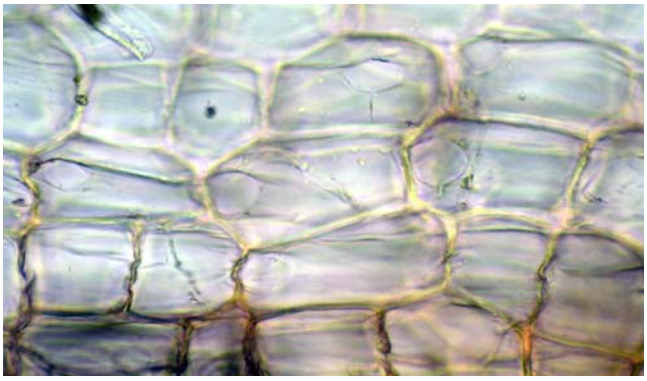

Figure 30. ***Sphagnum russowii*** stem epidermis, with no stain. Photo by Rudi Zielman.

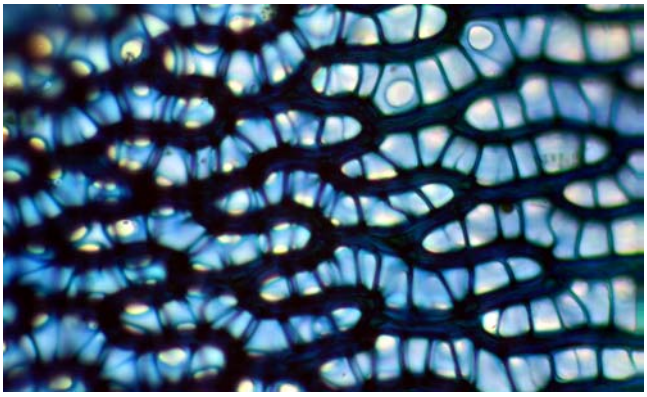

Figure 31. ***Sphagnum russowii*** leaf cells, stained with methylene blue. Photo by Rudi Zielman.

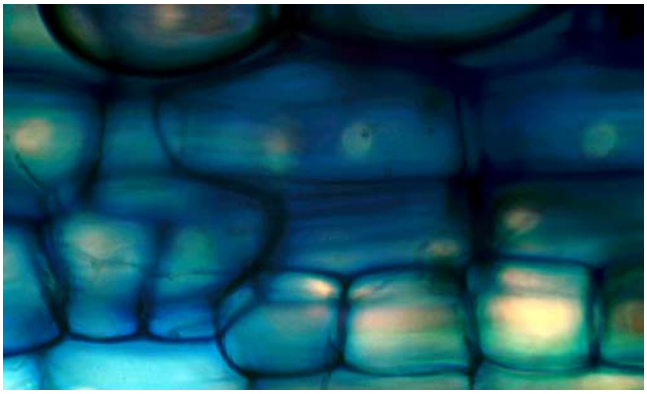

Figure 32. ***Sphagnum russowii*** stem epidermis, stained with methylene blue. Photo by Rudi Zielman.

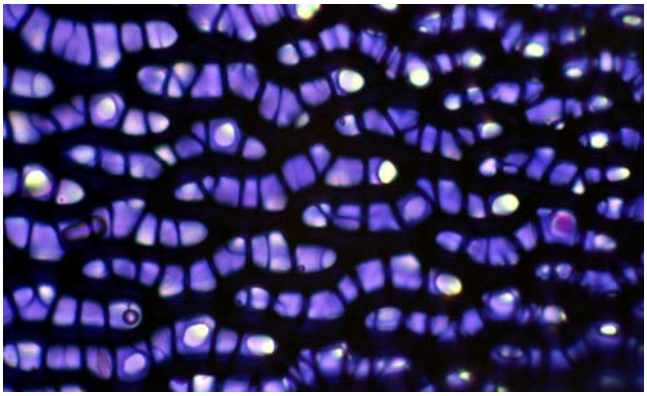

Figure 33. ***Sphagnum russowii*** leaf cells, stained with toluidine blue-O. Photo by Rudi Zielman.

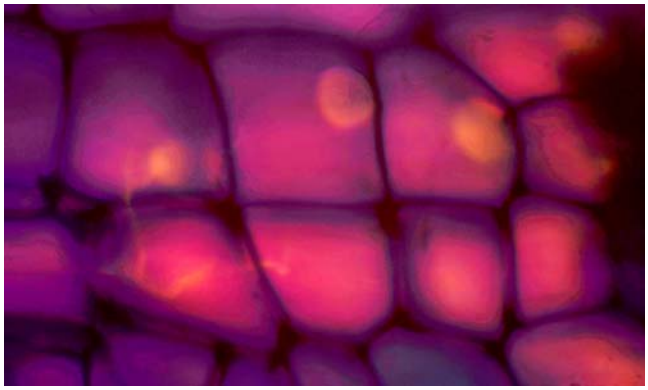

Figure 34. ***Sphagnum russowii*** stem epidermis, stained with toluidine blue-O. Photo by Rudi Zielman.

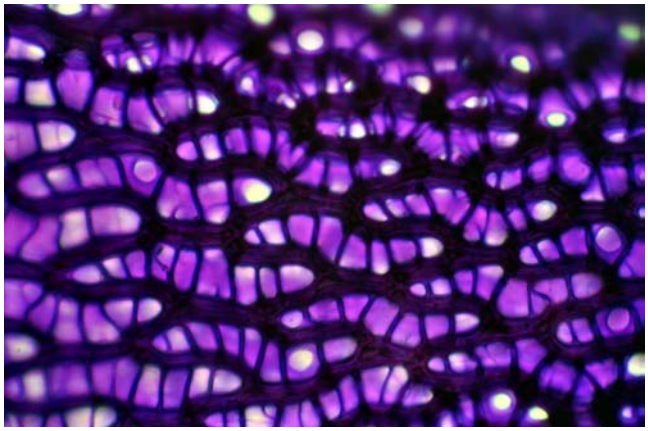

Figure 35. ***Sphagnum russowii*** leaf cells, stained with gentian violet. Photo by Rudi Zielman.

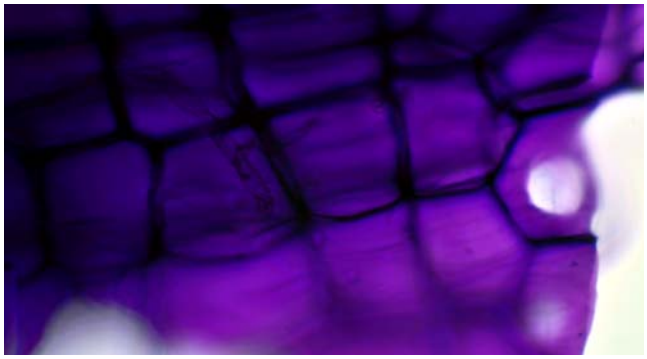

Figure 36. ***Sphagnum russowii*** stem epidermis, stained with gentian violet. Photo by Rudi Zielman.

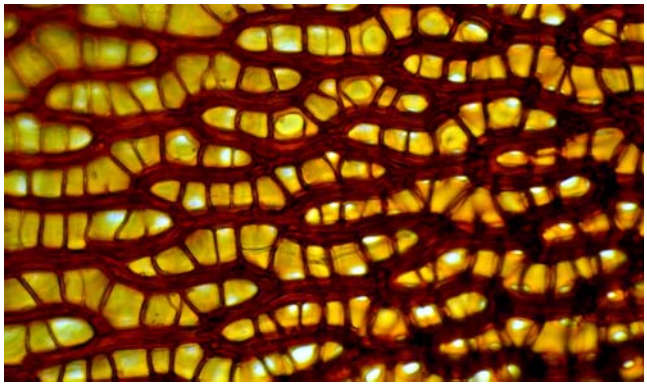

Figure 37. ***Sphagnum russowii*** leaf cells, stained with safranin. Photo by Rudi Zielman.

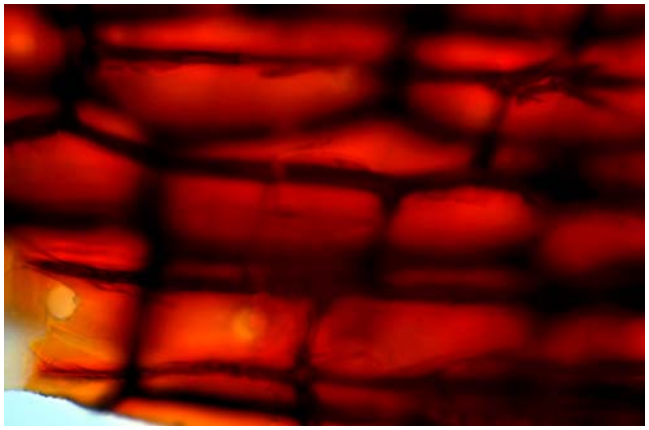

Figure 38. ***Sphagnum russowii*** stem epidermis, stained with safranin. Photo by Rudi Zielman.

Judgment Call

The staining of ***Sphagnum*** helps in making pores visible, as unstained gaps in stained walls, but is not always necessary. As an example, one can make a good judgment on ***Sphagnum divinum*** (Figure 6-Figure 16) and ***Sphagnum centrale*** (Figure 39-Figure 42) without staining. Differentiating these species depends on the thickening of the cell walls of chlorocysts as seen in section, most obvious on the adaxial (= ventral) leaf side. Staining can help in assessing this wall. In general, however, we recommend the location of chlorocytes to be assessed by unstained cross-sections.

Figure 39. ***Sphagnum centrale***. Photo by Hermann Schachner, through Creative Commons.

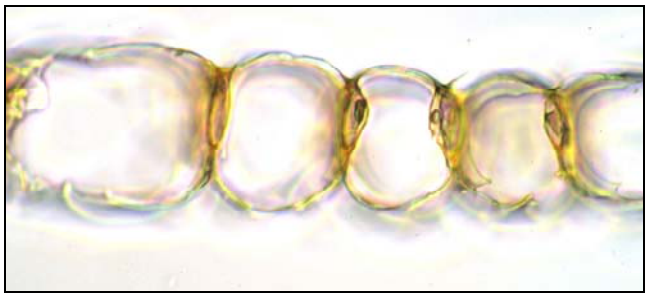

Figure 40. ***Sphagnum centrale*** unstained leaf cross section showing the almost hidden chlorocytes and thicker walls on the adaxial side of the hyalocytes. Photo by Rudi Zielman.

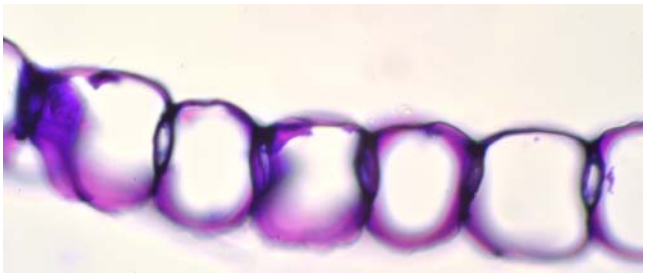

Figure 41. ***Sphagnum centrale*** leaf cross section with gentian violet stain. Photo by Rudi Zielman.

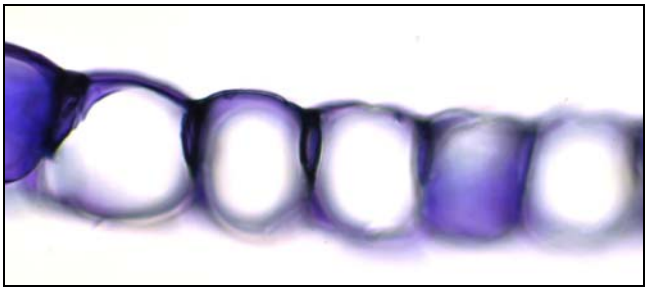

Figure 42. ***Sphagnum centrale*** leaf cross section with toluidine stain, giving a slightly better view of the thicker adaxial walls of the hyaline cells than in the gentian violet stain. Photo by Rudi Zielman.

If a decision has to be made as to whether faint pores or pseudopores are at hand, staining must be used. The dye which is used is less important as long as it is a cationic dye as already noted by Daniels and Eddy (1990). If I (Zielman) had read this in 2012 more carefully… For the rest it is merely a matter of taste; Adam Hölzer (2010) only wants to use gentian violet; Lisa op den Kamp (Bryonet, October 2012) has a strong preference for safranin; this is also the stain that was used by Laine *et al*. (2009), whereas in Australia all staining of botanical tissue is standardly done with toluidine blue (Rod Seppelt, pers. comm.). In general methylene blue, that used to be widely used, is considered a staining which is uncomfortably harsh and dark. Because of this I am, for the ease of use and availability, using more and more safranin, after my first bottle of gentian violet was empty. But after completing the work for this article, I tend to use toluidine blue, although it is tricky to obtain, or gentian violet. All in all it remains a tad a matter of personal taste, so not a firm conclusion. But I am going to use stains more often again, for an easier assessment.

Summary

Sphagnum pores are usually difficult or impossible to distinguish in unstained material. Some stains in use in the last century are toxic. And some current ones are difficult to obtain. Among the ones tested here, safranin and methylene blue are both safe and available from internet sources, gentian violet or toluidine blue might be preferred if obtainable. Some of the staining solutions can be made from powders, but it is easier to just buy the solutions ready-made.

Acknowledgments

This chapter is based mostly on a publication in Dutch (Zielman 2020). We all owe Rudi Zielman a vote of thanks for documenting the differences among the available stains.

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CHAPTER 2-3
LABORATORY TECHNIQUES: MAKING OBSERVATIONS

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CHAPTER 2-3
LABORATORY TECHNIQUES: MAKING OBSERVATIONS

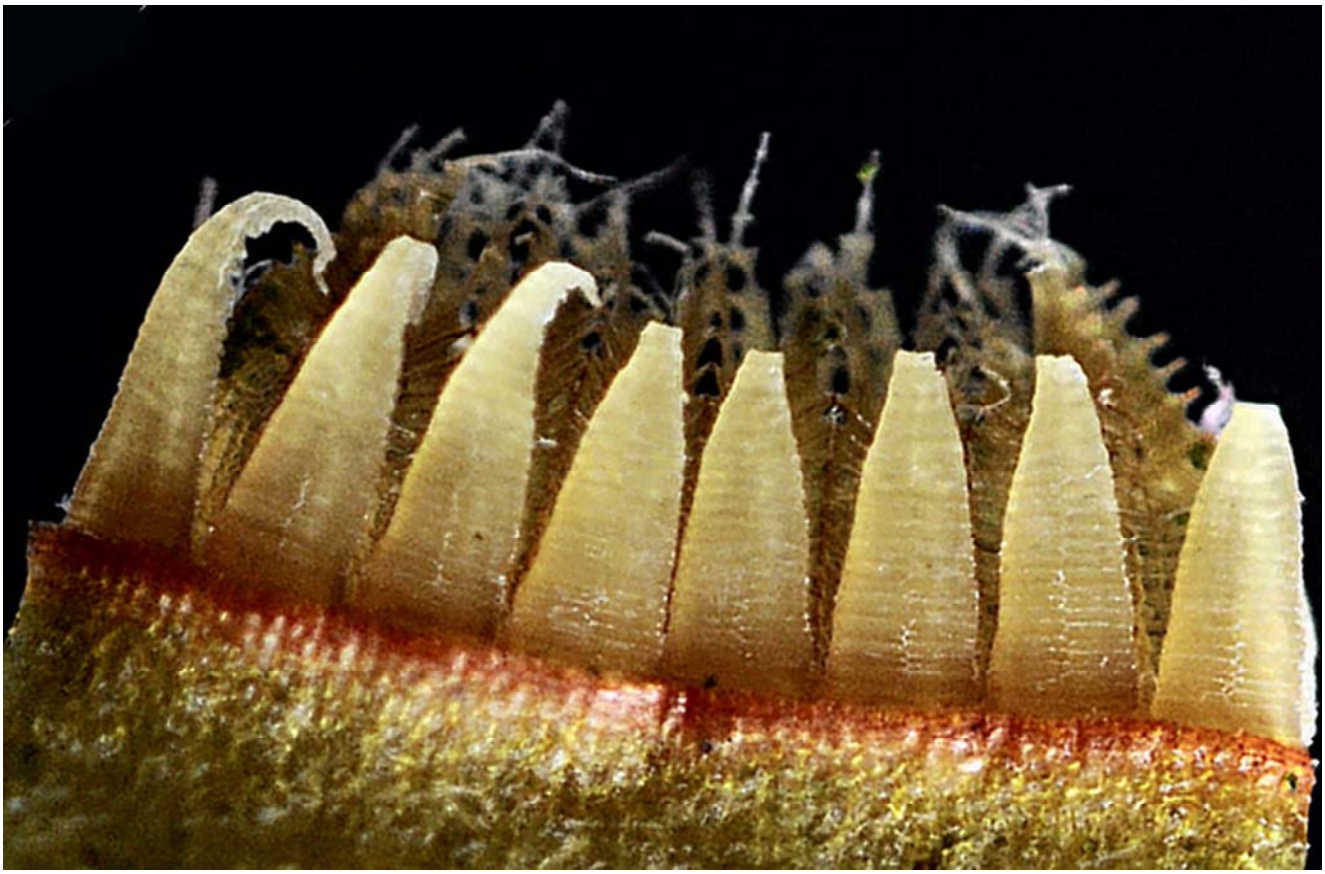

Figure 1. ***Plagiomnium rhynchophorum*** peristome ready to perform its dispersal of spores. Photo by George Shepherd through Flickr.

Sporophytes

Stomata

One of the most difficult things to see on a moss is the stomata at the base of the capsule. Rod Seppelt (Bryonet 27 August 2012) suggests clearing the capsules with lactic acid to make them easier to see. This method works well also to make it easier to see cell walls, cell content, papillae, and exothecial cells, including those on liverworts as well as mosses. The material should first be wet on a microscope slide and covered with a coverslip. Then place a drop or two of lactic acid on the edge of the coverslip and allow it to diffuse into the mount. **Gently warm the slide over a spirit flame** (a gas flame is too hot); this mix boils very quickly. The lactic acid gets rid of most of the cytoplasmic contents, thus clearing the tissues. If you want to make the mount permanent, then you must be sure all the lactic acid has been removed by heating, then add a permanent mounting medium at the edge of the coverslip.

Opening Immature Capsules (Lauridsen 1972)

Removing an operculum while keeping the peristome intact is difficult to impossible. Lauridsen (1972) tested a method that "fools" the capsule into behaving as if it is ripe, releasing the operculum. This is done with alternate immersion in **KOH** and **NaOCl**. The amount of KOH and NaOCl varies with species and ripeness and needs to be tested each time. This is best accomplished by dousing the capsule first with a drop of NaOCl for a few seconds, then in a solution of KOH until the mouth of the capsule becomes reddish. This may take 10 sec – 3 minutes. Zander (1993) recommends 2% KOH for Pottiaceae.

Although the method did not work well with ***Funaria hygrometrica*** (Figure 2) (14% opened), it was more than 90% successful in ***Bryum argenteum*** (Figure 3), ***B. intermedium*** (Figure 4), ***Distichium inclinatum*** (Figure 5), ***Mnium hornum*** (Figure 6), and ***Polytrichum commune*** (Figure 7) (Lauridsen 1972). The biggest disadvantage of the method is that the peristome teeth and exothecial cells

both become reddish in KOH, and the coloration remains. If possible, some capsules should be kept intact with no chemicals to retain natural colors. The treatment and color changes should be noted on the packet so as not to confuse further researchers with the altered colors. These chemicals should be washed away before mounting the specimen in Hoyer's or returning them to the packet. KOH plus Hoyer's will present a white precipitate, and excess NaOCl will eventually discolor the capsules.

Figure 2. ***Funaria hygrometrica*** capsule, with a lid (operculum) that is hard to remove. Photo by George Shepherd, through Creative Commons.

Figure 3. ***Bryum argenteum*** capsules, with opercula easily removed with KOH. Photo by Dick Haaksma, with permission.

Figure 4. ***Bryum intermedium*** with capsules; the operculum in this species is easily removed with KOH. Photo by David T. Holyoak, with permission.

Figure 5. ***Distichium inclinatum*** with capsules; the operculum in this species is easily removed with KOH. Photo by Michael Lüth, with permission.

Figure 6. ***Mnium hornum*** with capsules; the operculum in this species is easily removed with KOH. Photo by J. C. Schou, with permission.

Figure 7. ***Polytrichum commune*** capsules; the operculum in this species is easily removed with KOH. Photo by David T. Holyoak, with permission.

Zander (1993), working with **Pottiaceae**, suggests that if the operculum does not come off, the slide can be heated with a butane cigarette lighter, taking care not to let the flame touch the glass. A coverslip will reduce evaporation. If the peristome has a habit of breaking at the base, soak the intact capsule in a mix of **KOH** and **Pohlstoffe** for 15-20 minutes, or longer. To avoid precipitation that occurs with Pohlstoffe in KOH, one alternative is to add 1-2 drops of concentrated (4 g in 20 cc water) stock solution of **sodium N-lauroylsarcosine** (Gardol) to the bottle of KOH instead.

Britton (1890) found that capsules did not retain their ability to expand when rewet after drying, so she suggested that they should be kept in a moist dish under cover until needed for observation.

Peristomes

Miller (1988) reminded us of both the beauty and interesting behavior of peristome teeth (Figure 1). He advised that to see the details of the peristome, split the newly opened capsule lengthwise. Mount half in water with the outside surface upward and the other half with the inside surface upward. Adjust the light on the microscope, using the **diaphragm**, to get the best view of the details.

Sean Edwards (pers. comm. 22 April 2014) excites his students by demonstrating what he calls the karate-chop method, using a ***Polytrichum*** peristome (Figure 8). Using this method, he distinguishes peristomes in three species of the **Polytrichaceae**.

Figure 8. ***Polytrichum*** epiphragm showing peristome teeth. Photo by George Shepherd Creative Commons.

To observe the hygroscopic movement of the teeth, Miller (1988) suggested removing the sporophyte with its seta intact. Thread the seta through a pinhole in a stiff piece of paper to position the capsule firmly in a vertical position. In some mosses, like **Mniaceae**, you can see movement of an exposed peristome (Figure 9) and spore dispersal by breathing on the dry peristome, providing a change in moisture. Observe the teeth at 40X and 100X while blowing moist breath across the teeth. This works best when a second person supplies the breath so that you can observe it with the microscope at the same time. This will only work well if the capsule is mature and the spores are still inside, but the peristome can still respond even if the spores are gone.

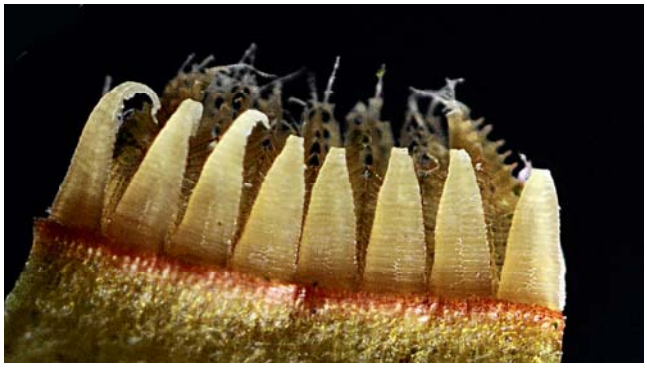

Figure 9. ***Plagiomnium rhynchophorum*** (**Mniaceae**) peristome – one that flexes in response to humidity in one's breath. Photo by George Shepherd, with permission.

I have had success in observing peristome movement with ***Dicranella heteromalla*** (Figure 10-Figure 11) by keeping the capsule and seta attached to the moss. The moss needs to be rehydrated by placing one or more drops of water on the leaves near the seta insertion. Exercise caution to avoid getting water on the seta or capsule. The nearby moisture first causes the seta (Figure 10) to gyrate, delighting the students; then the peristome teeth (Figure 11) begin to flex. Breathing on it might give the same result.

Figure 10. ***Dicranella heteromalla*** with capsules; setae in this species will gyrate in response to moisture changes. Photo by Bob Klips, with permission.

Figure 11. ***Dicranella heteromalla*** capsule showing peristome teeth that will respond to moisture changes. Photo from Botany 321 Website, UBC, with permission.

Anchoring Specimens in Clay

Conard developed a unique idea for examining peristomes and their activities. He suggested placing a bit of clay (any color) 5-8 mm across and 1-2 mm thick on a slide. A capsule, with its seta removed, can be placed in any desired position for observation with high power on a compound microscope. The right clay will remain soft, so the slide can be kept for several years and the capsule can still be repositioned. This can also be useful for demonstrating peristome movement to students and for other uses where positioning is important.

Counting Spores

Britton (1890) detailed a way to examine the capsule and its contents. She suggested that observing a dry capsule on a microscope slide under low power on a compound microscope (4x or 10x objective) could lead to the breaking of the annulus that holds the lid (operculum) to the capsule. If the annulus releases the lid, the dryness will cause capsule compression and spores will be pushed out. If there are still too many spores in the capsule, put a drop of water on one edge of the coverslip and draw it through with a piece of paper towel or blotter on the opposite edge. The spores will be drawn out as the water moves.

If this procedure is unsuccessful, you can encourage the spores to come out by holding the slide over the flame of an alcohol lamp until the water boils (Britton 1890). This drives the air out of the capsule and the spores with it. BE CAREFUL with this technique because if the slide gets too hot it can break, sometimes explosively. Withdraw the slide before the water dries up completely.

Most of the recent spore counting techniques have been copied from pollen counts. To obtain a sense of variability, one capsule is not enough, despite the large number of spores in most species. Fifteen is a reasonable number, but they should, if possible, be distributed among 15 clumps to minimize the bias of a single genotype. One drop of Extran® in 5 ml distilled water can prevent clumping. These can be spread in 5 ml water in a **Newbauer chamber** (used for counting platelets and red cells in blood). This chamber is designed for a thick crystal slide with the size of a glass slide (30 x 70 mm, but 4 mm thickness). The counting area is located in the center of the slide. Counting can be done at 100x, with further replication achieved by four counts per sample. The mean number of spores per sporangium is used. Size uses the greatest diameter and can be based on photomicrographs analyzed with ImageJ software (Rasband 1997-2002).

Spores can be difficult to observe because of their density. Miyoshi (1969) compared the visibility of the special surface ornamentation under light and SEM microscopy, demonstrating the superiority of SEM for this purpose. Other methods are covered in Chapter 2-2 of this volume.

David Wagner (Bryonet 22 January 2020) suggested a method that is used for pollen grains in anthers. The anther or capsule is wetted with a small amount of wetting agent. This keeps the spores in a clump instead of floating to the edges of the drop. Smash the capsule in a small drop of glycerine that will fit under the coverslip. Instead of a normal coverslip, use a **reticle** (series of fine lines or fibers in eyepiece of optical device such as microscope, used as measuring scale or aid in describing location of objects) with a 1 cm grid marked in millimeters. A grid of this size will have one square visible under the 10X objective of a compound microscope. This magnification is high enough to count the spores one square at a time, from top right to lower left, 100 squares. It is tedious but gives a very accurate result with a single mount.

Efrain De Luna (Bryonet 22 January 2020) suggested that to avoid the tedious stem, you can use the NIH Image (ImageJ, free software) or purchase ImagePro (Media Cybernetics) to recognize such discrete objects as cells, spores, *etc*. and determine their size and counts with the software.

Tom Ottley (Bryonet 22 January 2020) suggested a more mathematical approach.

Wagner assumes that evolutionary selection would result in the maximum number of spores being packed into a capsule. Then one can measure the internal diameter of the capsule and the diameter of a spore. Capsule diameter, divided by spore diameter, cubed x 0.6 would give the number of spores. He then multiplies by 0.5 to compensate for other structures (such as the columella) taking up space. This should actually be calibrated for each species by comparing to actual counts, and spores would probably need to be at the same stage of development. Ottley also suggesting that weighing a few (~10) ripe capsules, then emptying the spores and reweighing the capsule could give you an estimate of the spore weight. You could calculate the weight from a measured diameter by assuming a density of 1. This requires a sensitive balance.

Nicholas McLetchie (Bryonet January 2020) suggests the alternative method of using a hemocytometer, following methods for counting blood cells.

One method for calibrating counts, used by palynologists, is to purchase a tablet with a known number of *Lycopodium* spores – usually with ~10,000 each (Bent Vad Odgaard, Bryonet 22 January 2020). One tablet is added to the solution containing the liverwort spores and a few drops of HCl are added to dissolve the calcium carbonate that that holds the tablet together. Since you know the number of *Lycopodium* spores, and if you assume an equal and even dispersion of both kinds of spores, you can compare the counts of the two kinds of spores under the field of view and use the ratio to calculate the total number on the slide.

Adam Hölzer (Bryonet 22 January 2020) suggests putting spores of several capsules in a measured amount of water (50 or 100 ml) with some glycerin. You will need to test several amounts of glycerin to find the appropriate amount. Then shake the mix very well and quickly remove 1 or 0.5 ml to a slide before the spores can settle. These can then be counted by one of the above methods.

Surface ornamentation and shape are likewise important in examining spores (Kristian Peters, Bryonet 13 November 2019). Some of these ornamentations are important in dispersal and may differ between aquatic and terrestrial species.

Flotability can also be an indication of density. Misha Ignatov (Bryonet, 12 November 2019) reported that he had heard about one experiment where **Polytrichaceae** spores remain floating on the water surface despite various

attempts to sink them, but after adding TRIS (reducing surface tension) they all sank immediately. Thus, their density is slightly greater than 1. Using solutions of various density one might like find out spore density quite precisely, if necessary.

Spore diameter is provided in most descriptive bryophyte floras for each species (Misha Ignatov, Bryonet 12 November 2019). For a description of shape, size, and ornamentation of moss spores in Europe, see Boros and Járai-Komlódi (1975).

Spore Dispersal

Place mosses or liverworts with mature capsules where the heat of a lamp is focused on them. Allow the bryophytes and their capsules to dry with the heat until the operculum comes off (mosses) or the capsule splits (liverworts). If it is a moss, the peristome teeth will begin to move as the capsule dries further and the seta may begin to gyrate. If it is a liverwort, the elaters will begin to twist, aiding in the dispersal of the spores.

This demonstration could be even more interesting by placing the capsule on an agar plate (see chapter on culturing) for the above procedure (we haven't tried this, so it might not work). Set the capsule into the agar so that it is upright. It might be necessary to put a narrow cellophane collar around it to keep the capsule from absorbing moisture from the agar. When spores disperse, they will land on the agar. The plate can then be covered to allow the spores to germinate.

Another method for determining dispersal distance is to place a capsule upright by one of the methods described earlier and place microscope slides coated in glycerine at designated distances from the capsule. The spores that are dispersed will be trapped by the glycerine and can be observed under the microscope. This could likewise be done with plates of agar. I would suggest the small Petri plates (35 or 50 mm) to save agar and space. This same technique will work in the field as well as in the lab. There will undoubtedly be contamination, but since the goal is only to locate spores and the distance travelled, contamination need not be a concern.

Living spores in the capsule or elsewhere can be distinguished from dead ones by several techniques. Fluorescence (see Chapt. 2-2 in this volume) will make living spores and living parts of spores visible when viewed using a UV light source. When using an ordinary light microscope, living spores can be distinguished using acetocarmine stain (Mogensen 1978). Living spores stain deep red, whereas dead spores do not stain at all.

Sperm

The first problem for observing sperm (Figure 12) is finding the antheridia at the right stage. By the time the male inflorescence is distinguishable, the sperm are likely to be dispersed (Jeff Duckett, Bryonet 11 January 2012). While many bryophytes are adapted to take advantage of spring rains for dispersal of sperm, we are learning that mites and springtails can disperse them, and some bryophytes take advantage of autumn water. ***Polytrichum*** (Figure 13-Figure 14) typically disperses sperm in early spring, ***Sphagnum*** (Figure 15) in autumn (Jeff Duckett, Bryonet 11 January 2012). ***Pellia*** (Figure 16), which has the largest sperm, disperses in early summer. Reese (1955) suggests that sperm are best collected during a dry period because rain will cause them to disperse and you will miss them. Of course if you see new growth arising from the antheridial head, you have missed the dispersal event (Figure 13).

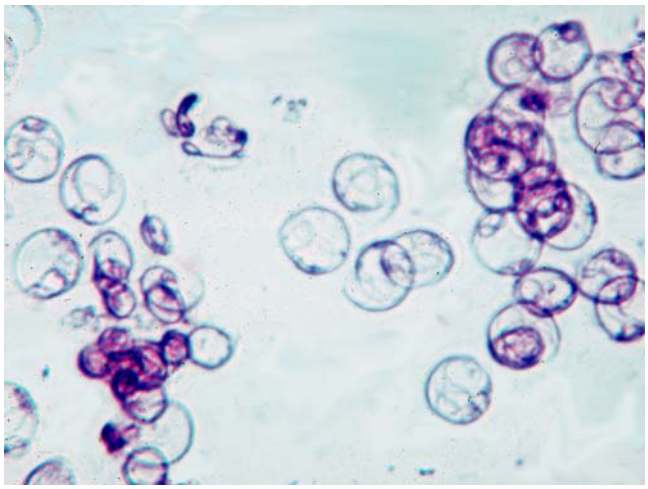

Figure 12. Stained bryophyte sperm. Image modified by Janice Glime.

Figure 13. ***Polytrichum juniperinum*** with splash cups that display new growth and hence have no viable antheridia in them. Photo by Janice Glime.

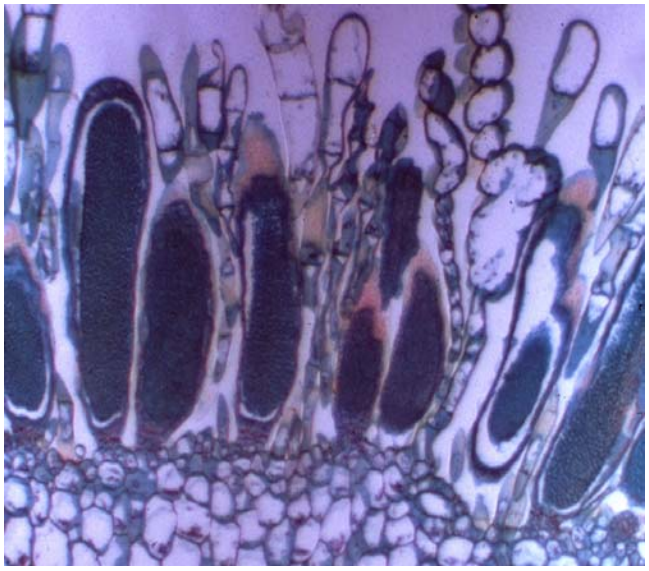

Figure 14. ***Polytrichum*** antheridia, where sperm are produced. Photo by Janice Glime.

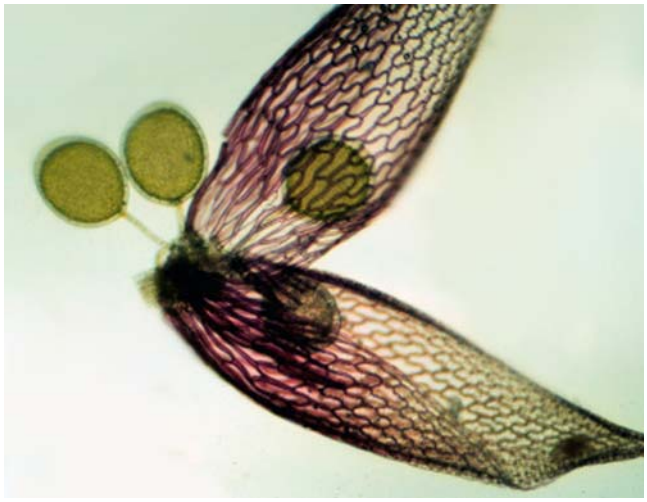

Figure 15. ***Sphagnum*** antheridia; these release sperm in autumn. Photo courtesy of Yenhung Li.

Figure 16. ***Pellia neesiana*** antheridia; these release large sperm in early summer. Photo by Michael Lüth, with permission.

If you are lucky enough to find ripe antheridia, you may need special techniques to actually see the sperm. First, you will probably need to squash the antheridia to release the sperm, a feat you can accomplish by pressing lightly on the coverslip, preferably while observing the antheridia through the microscope so you don't overdo it.

Once you have freed the sperm, they may not be as easy to observe as you might expect. They are in constant motion, so it might help to add a bit of **methyl cellulose** to the medium to slow them down (Rod Seppelt, Bryonet 11 January 2012). Even so, they are transparent, eluding detailed observation. Use the diaphragm of your microscope (NOT the rheostat) to decrease the light and increase contrast.

Reese (1955) presented a method for observing sperm. He suggested clipping off the antheridial heads or branches with perigonia and inverting several in a drop of water on a slide. The source of water is important, with chlorine in tap water killing the sperm, and distilled water likewise having deleterious effects, perhaps causing the cells to take in water and explode. Reese suggests letting tap water sit overnight. The slides can be put aside in Petri dishes with damp filter paper until the water on the slide becomes milky, indicating that spermatozoids have been released. At this point, the antheridial parts should be removed, leaving only water and sperm on the slide. Set the slide somewhere to dry in preparation for staining. The dry sperm can be stained with a 1% aqueous solution of gentian violet. You can immerse the slides in the gentian violet or place a few drops of the stain on the slide. All that is needed is 30-60 seconds to stain the material. Then wash the slide with distilled water and de-stain it in 50% ethanol for 10-20 seconds. Wash it again in distilled water, allow it to dry, and mount it in your choice of mounting media.

To observe live antherozoids, Reese (1955) recommends smearing a small amount of fresh egg albumen on a slide and adding a drop of water with freshly discharged antherozoids. You can add a cover slip if you wish to observe. The albumin helps to slow down the movements of the antherozoids. Using darkfield illumination helps in observing these, or close the diaphragm down as far as it will go.

Des Callaghan has created a film that shows sperm in motion <http://youtu.be/Jdh8flxvZgk>. These were not stained, but instead used differential interference microscopy (DIC) to create the contrast needed for the sperm to be visible.

If you just want to find sperm, and possibly count them, you can probably succeed with Sperm VitalStain™. We have not tried this – it is designed for human sperm and it can distinguish between living and dead sperm. The stain contains both eosin and nigrosine. The eosin will be absorbed by the dead cells – those with a damaged plasma membrane – and will stain these cells red. Nigrosine is a counterstain that facilitates the visualization of the living (unstained) cells. Instructions are available on their web page.

Nelly Horst (pers. comm. 3 February 2013) reports that DAPI staining (available from chemical suppliers) works nicely as a stain (Figure 17).

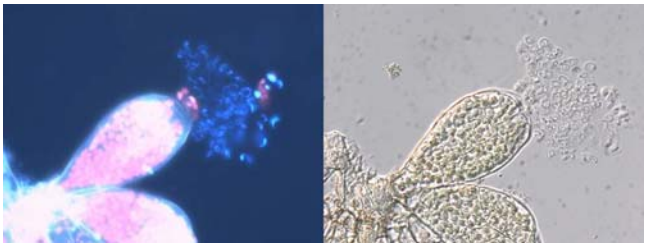

Figure 17. Sperm with DAPI stain (**left**) compared to fresh material (**right**). Photos courtesy of Nelly Horst.

Paraphyllia

Seeing these small structures can be challenging, even in large species. Rod Seppelt (Bryonet 26 October 2022) offers suggestions to make it somewhat easier: For **pseudoparaphyllia**, he finds that by using a pair of very fine forceps (*e.g.* Watchmakers No. 5, but they are about AU$50 in Australia) it is possible to press slightly on the stem and at the same time clasp the base of a branch and tear/slice away the branch base. This will have a bit of the stem and, with any luck, the pseudoparaphyllia if they are present. It is a bit frustrating, however.

Axillary Hairs

Seppelt (Bryonet 26 October 2022) relates that early on he asked Bill Buck where to look for axillary hairs. The reply was to look at the very apex of the shoots. He may have been primarily referring to pleurocarps. But, in general terms, carefully remove a number of leaves, clear the leaves in lactic acid (gets rid of the cellular contents making cell details more easily visible), and then go hunting, examining the basal attachment part of the leaves. They are often very small, sometimes with 1-2 tinted (yellowish or brownish) basal shorter cells. Length varies and it is a moot point as to what constitutes a "mature" axillary hair. Seppelt has also found that axillary hairs are not always to be found in the shoot apices. In some ***Fissidens*** (Figure 18) species, for example, they can still be found in the axils of leaves several pairs of leaves below the shoot apex.

Figure 18. ***Fissidens adianthoides***; in some species of ***Fissidens*** one can find axillary hairs at some distance below the apex. Photo by Hermann Schachner, through Creative Commons.

Jeff Duckett (Bryonet 27 October 2022) has found that the best way to see axillary hairs is to cut the stem apex longitudinally and squash it with the cut surfaces uppermost. This will make the hairs readily visible in the axils of the young leaves. These hairs are short-lived and thus one is unlikely to find them among older leaves.

Hedenäs (1989) examined the axillary hairs of 200 pleurocarpous moss species. He pulled the leaves off the terminal ~5 mm of the stems. He found that the branches usually have fewer and weaker axillary hairs. Hairs could often be found attached to the bases of detached leaves where stem portions remained, but in other cases it was necessary to squash the tips.

Leaf Movement

Beginning students are often in awe when they drop water onto a moss like ***Hedwigia ciliata*** (Figure 19-Figure 20). The leaves spread before their eyes like a well-orchestrated ballet. Place a moss branch on a glass slide or in a Syracuse watch glass and add water to one end. As students watch the leaves spread, this permits a good discussion on why. They can compare species and further investigate to try to determine why some spread more easily than others.

Hedwigia (Figure 19-Figure 20) is great for a spreader (Figure 20), **Mniaceae** for non-spreaders (Figure 21) (without special coaxing). They can compare this behavior to that of dry tracheophyte leaves. It is an interesting exercise to try to determine what mechanism causes the leaf spread. For example, in ***Polytrichum*** species (Figure 13), the large, non-chlorophyllous area at the base of the leaf (Figure 22-Figure 23) fills with water and forces the leaf out. One can compare a variety of species and examine the leaf structure to see what facilitates the movement. This simple exercise can lead to lots of questions and simple observations and experiments – role of temperature of the water, other liquids, leaf structure (alar cells, borders, costa), staining to track where the water goes, etc.

Figure 19. Dry ***Hedwigia ciliata***. Photo by Des Callaghan, with permission.

Figure 20. Wet ***Hedwigia ciliata***, showing spreading of the leaves. Photo by Li Zhang.

Figure 23. ***Polytrichastrum alpinum*** (**Polytrichaceae**) leaf base showing hyaline cells and absence of lamellae. Photo by Dale A. Zimmerman Herbarium, Western New Mexico University, with permission.

Figure 21. ***Plagiomnium*** branch resisting rewetting. Photo courtesy of J. Derek Bewley.

Figure 22. ***Polytrichum juniperinum*** showing leaf base that lacks lamellae (arrow) and illustrating the spreading of hydrated leaves. Photo by Janice Glime.

Water Movement

Water enters bryophytes in various ways. For the majority of bryophytes, entry is through the leaves and in at least some it occurs most easily at the tips of the stems. But for some it is able to enter through rhizoids, and others absorb water throughout the plant. Once water gains access, various structures can help to move the water more quickly. Hydroids in stems seem to function like tracheids and vessels, moving the water upward in the plant through elongate capillary spaces of the hydroid cells. Alar cells can facilitate entry of water at leaf bases and from there it might enter the costa, travelling more quickly through the elongate cells of the costa because it has fewer cell walls to cross. But water will also move across the leaf lamina from cell to cell. And in mosses like ***Polytrichum*** spp., there are leaf traces (Figure 24) that can facilitate movement of water from the stem into the leaf. There is little published data to demonstrate how each of these structures affects the speed of movement and how that differs among taxa. Of even greater interest is a comparison of these structures and their effects on water movement as it relates to habitat.

Water movement can be demonstrated with dyes placed at various positions on the moss (Figure 25). Dyes at the tip can demonstrate how far downward the stain is able to go in a period of time and how it gets there – central strand? stem cortex? leaves? external capillary spaces? Plants positioned with their rhizoids in a dye can demonstrate the ability of rhizoids to take up water and the ability of the plant to move it upward.

After the dye has been placed on the portion of the plant of interest, one can cut sections at intervals to look for the presence of the dye in various parts of the plant. Care must be taken to prevent external dye from reaching other tissues when the specimen is mounted on the slide, so it is best to apply the dye, permit it to enter the plant for the time desired, then thoroughly wash the outside of the plant until it no longer discolors the wash water.

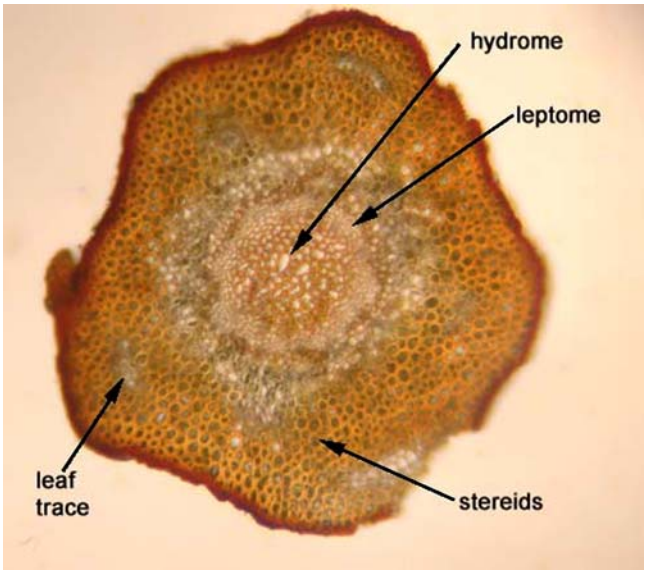

Figure 24. ***Polytrichum commune*** stem cross section showing leaf traces. Photo from UBC botany website, with permission.

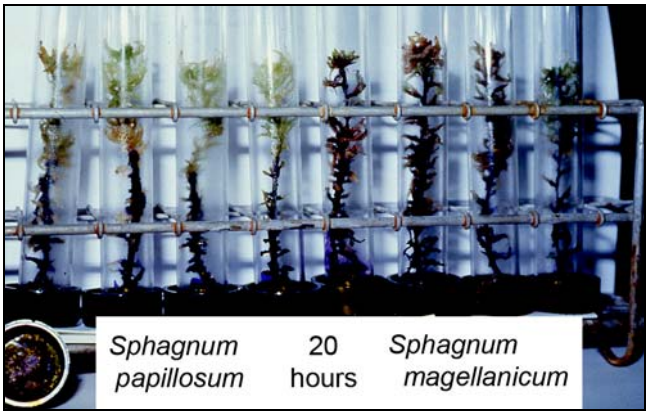

Figure 25. Demonstration of external water conduction in two species of ***Sphagnum***. Note that it has travelled much farther in ***Sphagnum magellanicum*** than in ***S. papillosum***. Photo by Yenhung Li.

intensities to see what that particular bryophyte responds to. Studies on bryophyte tropisms are limited (See Volume 1, Chapter 5-5), so new discoveries are almost certain.

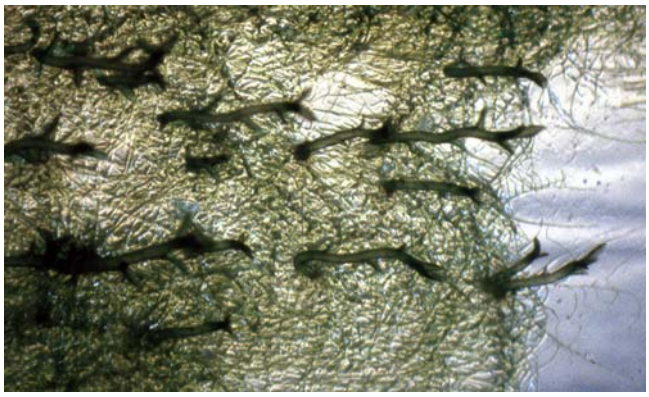

Figure 26. Phototropism of ***Funaria hygrometrica*** with light coming from right side of Petri plate. Photo by Janice Glime.

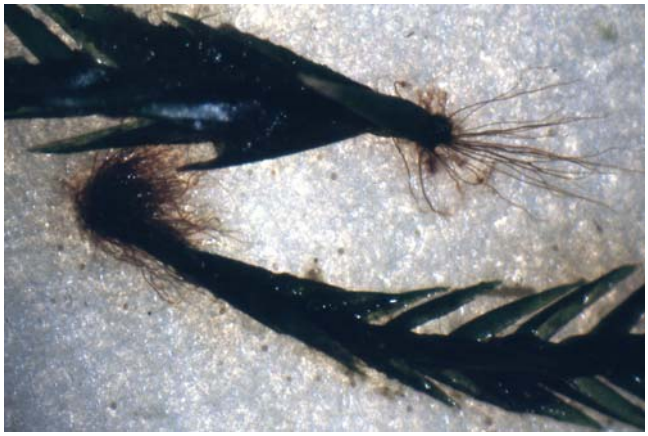

Figure 27. ***Fontinalis squamosa*** rhizoids exhibiting negative phototropisms to light coming from the left. Photo by Janice Glime.

Tropisms

Most, perhaps all, mosses exhibit tropisms. But do any, or all, liverworts do the same? Tropisms can be exhibited by culturing spores and using gravity (in darkness) or light from one side to test for these two tropisms (Figure 26-Figure 27). But a simpler method is to use a plastic bag and arrange the bryophytes to change direction of gravity or light. If testing for effects of one of these, be sure that the other cannot have any effect. For gravitropism, the clump can be placed on its side and light excluded. For phototropism, the clump should remain in its normal upright position and light should come from one side (Figure 26). Figure 28 demonstrates a combination of light and gravity on mosses on agar plates to test which has the stronger effect. Once a phototropism has been observed, one can experiment with various colors of light and

Figure 28. Paper rolls used to test the combined effects of light and gravity on tropisms. Photo by Janice Glime.

Etiolation

Etiolation can be demonstrated by placing bryophytes in a sealed plastic bag and giving them almost no light, such as that in a desk drawer or cabinet with the door closed. Within a few days or a week your bryophyte is likely to become elongated and spindly. I (Glime) sometimes see this in my terrarium where mosses grow from spores and have less light intensity than outdoor light.

Splash Cup Dispersal

The distribution of gemmae from the gemma cup of ***Marchantia*** (Figure 29-Figure 30) can be shown, and this might be applied to other splash dispersal structures in bryophytes. The splashing is effected by dropping colored ink or food coloring from a titration column onto the splash cup. The liverwort should be surrounded by white paper. As the dye hits the splash cup from the titration column, it splatters around the splash cup and makes blue spots on the white paper. This is a minimal approximation of the ability of raindrops to splash the gemmae. The distance of the water dropping is much less than that of a real raindrop, hence not having the same impact and resulting in shorter splash distances. The person doing the "splashing" should wear an apron or other protection during this demonstration.

Figure 29. ***Marchantia polymorpha*** gemmae cups. Photo by Michael Lüth, with permission.

Figure 30. ***Marchantia polymorpha*** gemmae cups with a few gemmae escaping in the upper chamber. Photo by Walter Obermayer, with permission.

Determining Oicy

Determining whether sexual organs occur on one plant or on separate plants is not an easy task. As Roxanne Hastings (Bryonet 14 April 2014) asserts "In order to be certain one must tackle the problem with systematic thoroughness." Using more traditional methodology, Hastings states that you must ensure that your clump of mosses is complete, including all the basal attachments, in order to determine **cladautoicous** (with antheridia on separate branch of same plant) mosses. She recommends taking a large clump, soaking it, and spreading it out on a slide or Petri dish. Then carefully tweeze the stems apart and remove any stems that are not basally attached. (You can't tell if they are part of the same or different plant.) Such fragments are only useful if the plant is **gonioautoicous** (having male and female reproductive parts on the same branch).

Then the tedium begins. Take a single stem and use needle-nose forceps, starting at the base, to carefully pull back each and every leaf to examine carefully for reproductive structures. When you reach the stem tip, remove that stem from the clump and set it aside. Then repeat the process on the next stem and every stem/branch of the clump until both sexes are located or you are certain only one is present. Yes, it can take several hours to peruse only one clump! Unfortunately, finding only one sex by this method is not definitive. Male organs typically develop before female organs do, or you might just be unlucky in finding only one of the sexes on your branches. And beware of the dwarf males (see Chapter 3).

But there is another way. And it even works for plants that are not producing sexual material at the time. Using the rarely reproducing dioicous moss ***Drepanocladus turgescens*** (Figure 31), Hedenäs *et al*. (2016) developed a method using a female-targetting marker that was previously developed for ***Pseudocalliergon trifarium*** (syn. ***D. trifarius***; Figure 32) and ***D. lycopodioides*** (Figure 33). When male and female portions of ***D. turgescens*** were sequenced and amplified, this method was successful in consistently revealing differences between males and females at five sequence positions. Alas, this method is likewise time-consuming and complicated, but it is reliable.

Figure 31. ***Drepanocladus turgescens***, a dioicous species for which a female-targetting marker can identify the gender. Photo by Michael Lüth, with permission.

Figure 32. ***Pseudocalliergon trifarium***, a species for which a female-targetting marker can be used to determine sex. Photo by Michael Lüth, with permission.

Figure 33. ***Drepanocladus lycopodioides***, a species for which a female-targetting marker can be used to determine sex. Photo by Michael Lüth, with permission.

Brownian Movement

Motion within a cell can often surprise the observer, especially a beginner. If you see chloroplasts moving as a group in something approaching a circle, it is most likely **cytoplasmic streaming**. However, if the movement is more like a vibration, it is most likely Brownian movement. Molecules are in constant motion, and these bump structures like oil bodies, causing them to move. Smaller particles within the cell will most likely also be in motion, but are less obvious. Ken Adams (Bryonet 2 February 2012) explains that at the small dimensions of cells, the thermal collisions of molecules against oil bodies is unbalanced. Thus, in any instant the number of collisions on one side of the oil body exceeds that on the other side.

Jeff Duckett (Bryonet 2 February 2012) reminds us that cytoplasmic streaming is unlikely to cause motion of liverwort oil bodies because there is almost no cytoplasm surrounding them.

Plasmolysis

Cyndy Galloway (Bryonet) uses the moss ***Physcomitrium*** (Figure 34) to demonstrate plasmolysis to students. A fairly strong salt solution causes the protoplasts to look like little basketballs. However, she said that adding water for deplasmolysis caused the cells to take in water too rapidly, causing them to burst. Perhaps that could be solved with some tinkering – a lower salt concentration, and replacement by water with some amount of salt that would be close to isotonic. ***Fontinalis duriaei*** (Figure 35) demonstrates plasmolysis caused by a copper solution (Figure 36). Observations on plasmolysis can be an assessment tool for contamination by heavy metals and will most likely be useful for other types of pollution as well.

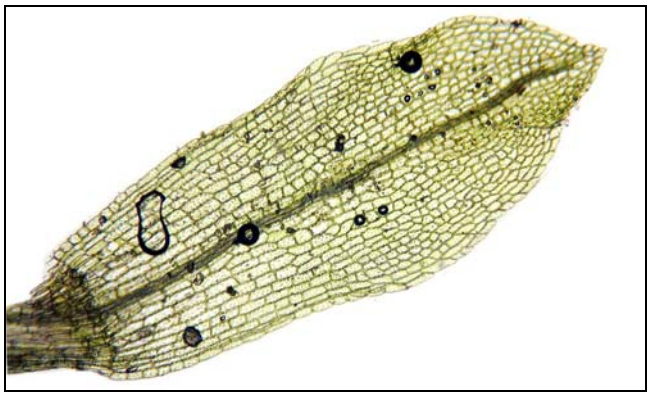

Figure 34. ***Physcomitrium sphaericum*** leaf cells, a good species for demonstrating plasmolysis. Photo by Michael Lüth, with permission.

Figure 35. ***Fontinalis duriaei***, a species that plasmolyzes in copper solutions. Photo by Michael Lüth, with permission.

Nutrient Cycling

There is a very easy, inexpensive exercise that can be done to demonstrate the role of bryophytes in nutrient cycles. Unfortunately, I don't know who contributed this, so if it is yours, please let me know!

Half fill a series of clear plastic (polycarbonate) cups with water – rainwater or distilled water is best. Add **methylene blue** dropwise to sets of three cups, one drop, two drops, and three drops for each cup in the set. (A small bottle of a concentrated solution of methylene blue can be obtained at a tropical fish store. It is a cationic stain that every bryologist should have on the lab bench.) The number of sets is determined by the number of samples.

Into each set place a moss or liverwort sample. Divide each sample into three parts. Add nearly equal amounts to each of the three cups in the set. A set = 3 cups, one with 1 drop, one with 2 drops, and one with 3 drops of stain. Volume by sight will work, but weighing is better. The sample should be small enough to be completely immersed in the methylene blue solution; bring the level up to about three quarters. It is interesting to compare nutrient sequestering abilities of ***Sphagnum*** (Figure 37), ***Eurhynchium*** (Figure 38), and forest floor leaf litter, for example.

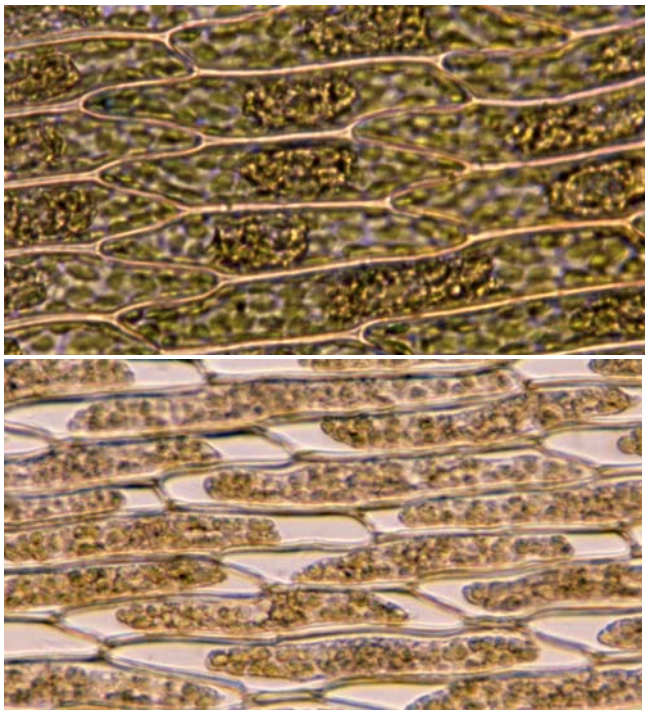

Figure 36. ***Fontinalis duriaei*** leaf. **Upper:** healthy leaf cells in water, demonstrating normal cell protoplasm arrangement. **Lower:** ***Fontinalis duriaei*** leaf cells in 100 µg L^{-1} copper, showing plasmolysis of cell contents.

Leave the sets overnight. The solution with moss will be clear in all of the cups (usually) while the dicot leaves and leaf litter water will still be blue. The graduated series of stain concentration helps visualize the effectiveness of the various samples. If you add an aquatic plant, you might get different results, so habitat comparisons could be quite interesting.

This demonstration shows how cations are scavenged by the cryptogams. In nature they pick out the micronutrients from rainfall that provide nutrition for the ecosystem or move them in solutions from the soil, using capillary spaces. Others remove them from water in streams and lakes. In industrial applications bryophytes clean water by picking out toxic metal ions and other cationic pollutants. This simple experiment can demonstrate rate differences among mosses and give an indication of their ability to extract nutrients from their ecosystems.

Figure 37. ***Sphagnum centrale*** with leaf litter. Photo by Janice Glime.

Figure 38. ***Eurhynchium oreganum***. Photo by Matt Goff, with permission.

Depending on the size of the cups, it may be necessary to start with a more dilute solution of stain than comes out of the bottle. Try adding lichens to the experiment for another comparison.

Measuring

Most of the material viewed using the microscope is too small to be measured with an ordinary ruler. Instead, we use a device called the **ocular** (eye) **micrometer** (reticule; Figure 39), which is a small disk that fits inside the eyepiece of the microscope. The disk has a tiny scale etched on it, and when we view a specimen, the image of the scale is superimposed on the object image.

The ocular micrometer scale is usually divided into units of 10 (Figure 39). To measure an object, simply count the number of units superimposed on the object. Thus the object shown in the margin is 10 **units** long.

Note that the measurement is reported in units, not microns or millimeters. We cannot assign a label to the units until the ocular micrometer has been **calibrated** (Figure 40-Figure 42). The scale needs to be calibrated with each objective on the microscope because the magnification of the scale never changes, whereas the magnification of the object does. An ocular micrometer placed in a 10X eyepiece is always magnified 10 times,

whereas an object under the objective is magnified by the power of the objective **and** the ocular.

To calibrate the ocular micrometer, you need to compare the scale with a scale of known dimensions that fits on the stage of the microscope (Figure 40-Figure 42). Remember that the stage is where all your organisms will sit on slides and perform for you (Figure 42). This special slide is known as a **stage micrometer**. It is a microscope slide with a tiny ruler etched on it. The marks on the ruler are exactly 0.01 mm apart (0.01 mm = 10 microns, µm).

Figure 39. Microscope ocular, showing where the ocular micrometer is inserted. Photo from Wikimedia Creative Commons.

Calibrate

1. Begin to calibrate the ocular micrometer by placing the stage micrometer on the stage and focusing on it with low power.
2. Move either the ocular or the stage micrometer until the two scales are superimposed.
3. Now move the stage micrometer laterally until the lines at one end coincide with each other. Call this point A.
4. Look for another line on the ocular micrometer that coincides with one on the stage micrometer. Call this point B.
5. Count the number of divisions on the ocular micrometer between points A and B.
6. Count the number of divisions on the stage micrometer between points A and B. Multiply this number by 0.01 mm to find the actual length of these divisions.
7. To find out how many mm equals 1 unit on the ocular micrometer, divide the answer to line 6 by the number of ocular micrometer units (line 5). (stage number of divisions/ocular number of divisions):

$$\frac{\text{mm}}{\text{ocular unit}} = \frac{0.01(\text{\# stage divisions})}{\text{\# ocular divisions}}$$

For example, in Figure 40 below, the number of stage micrometer divisions between points A and B is 6. The number of ocular micrometer divisions in this distance is 3. Therefore, the mm/unit on the ocular micrometer is:
(6 x 0.01 mm)/3 = 0.02 mm/unit or 20 µm.

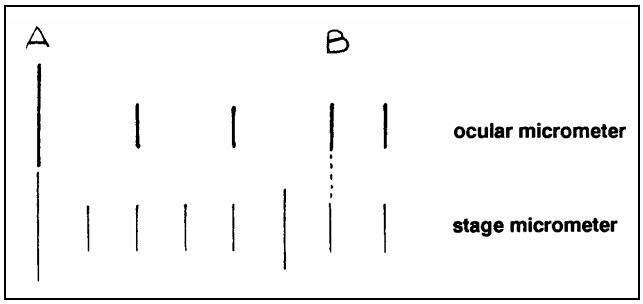

Figure 40. Alignment of scale of ocular micrometer (reticule) with that of the stage micrometer as seen in the eyepiece of the microscope. Drawn by Shelly Meston.

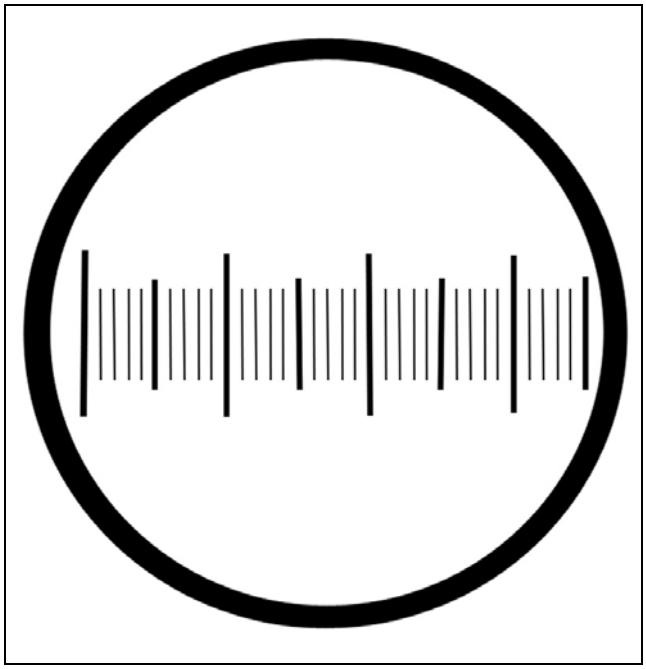

Figure 41. View of an ocular micrometer (reticule) in the eyepiece of the microscope. Drawn by Janice Glime.

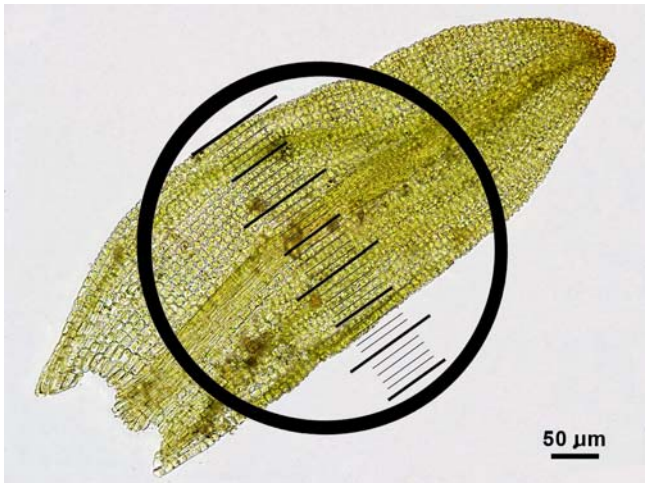

Figure 42. Reticule in position to measure width of midleaf of ***Barbula convoluta***. Measured width is 28 units and must be calibrated against stage micrometer. Leaf image by Dale A. Zimmerman; reticule by Janice Glime.

An object under this ocular and this objective measuring 8 units would be 0.16 mm long. Calibrate your ocular micrometer for each objective on your microscope and record the conversions below to keep as a reference.

low power:
stage micrometer units ____
ocular micrometer units ____
mm/unit ____

medium power:
stage micrometer units ____
ocular micrometer units ____
mm/unit ____

high power:
stage micrometer units ____
ocular micrometer units ____
mm/unit ____

oil immersion:
stage micrometer units ____
ocular micrometer units ____
mm/unit ____

You should also include the scale when you make drawings. Follow the instructions above for calibrating an **ocular micrometer**, which is a small ruler that fits in the eyepiece of your microscope. Using this ruler, you can measure things you see under the microscope. If you do not have an ocular micrometer available, you can estimate the size of objects using only a **stage micrometer**. The stage micrometer is a special slide that has a tiny ruler etched on it. Place the stage micrometer on the stage of your microscope and focus on the ruler with low power. Each mark on the micrometer is 0.01 mm apart. Use the ruler to measure the diameter of your field of view. Do this for each power on your microscope and record the diameters in the blanks below. Be very careful when focusing under the higher power objectives because the stage micrometer is very expensive. Then, use this information to estimate the size of objects seen under each power and to include a scale with each drawing you make.

field of view diameters:
low power ____
medium power ____
high power ____

Leaf Measurements

It is challenging to measure leaf cells because, unlike bricks, they are not rectangular. Rather, their sides are not parallel and their width and length change along the cell. In an attempt to solve this measurement problem, Ivanov and Ignatov (2011) developed a method to digitize the "cell net." Using this software, one can measure cell length, width, and area. They compared the published cell width for five moss species in five different publications (Table 1).

Table 1. Comparison of published cell width data for five pleurocarpous moss species in µm (from Ivanov & Ignatov 2011).

	Noguchi		Lawton		Ignatov & Ignatova
		Smith	Limpricht		
	1992	2004	1971	1885-1904	2004
Ptilium crista-castrensis	2	4-5	3-5	5	4-6
Callicladium haldanianum	4-4.5	-	5-7	6	5-8
Calliergonella lindbergii	3-4	5.0-6.5	4-7	6-7	5-7
Isopterygiopsis muelleriana	4-4.5	4-6	-	5-6	-
Hylocomium splendens	3-4	5-7	4-6	5	5-6

Later, Ivanov and Ignatov (2013) developed a 2-d digitization of plant cell aerolation using polarized light microscopy. This microscopic image is photographed into a digital photo. Using ***Plagiomnium elatum*** **(**Figure 43-Figure 44**)** and ***P. medium*** (Figure 45-Figure 46), they digitized the cell arrangement of the oblique rows (Figure 44) on these unistratose leaf lamina. They proposed a "computer analytic method that allows transferring visible images into coordinates of intracell boundaries and their **vertices** (points where three or more cells contact), *i.e.*, into a digital cell net. After such a digitizing it is possible to estimate many geometrical parameters of cells and their complexes under relatively simple mathematic treatment." Chemical or physical coloring methods should be used to permit distinction between cell boundaries and intracellular space. In some cases, this might be done by fluorescence microscopy. The method is somewhat complex, so I refer the reader to the original paper as I have not tried it myself.

Figure 43. ***Plagiomnium elatum***. Photo by Michael Lüth, with permission.

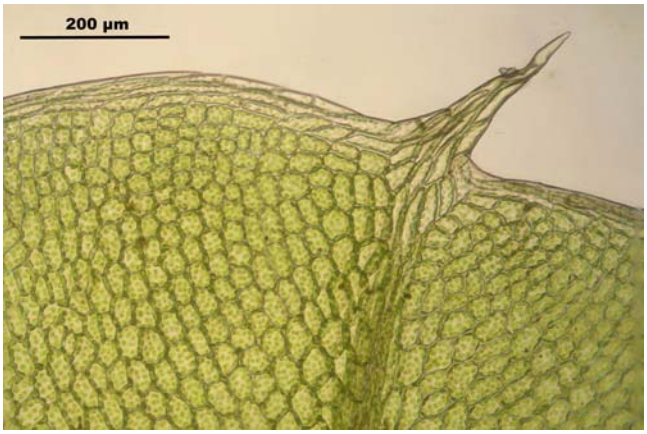

Figure 44. ***Plagiomnium elatum*** showing oblique rows of leaf cells. Photo by Hermann Schachner, through Creative Commons.

Figure 45. ***Plagiomnium medium***. Photo by Dale A. Zimmerman Herbarium, Western New Mexico University.

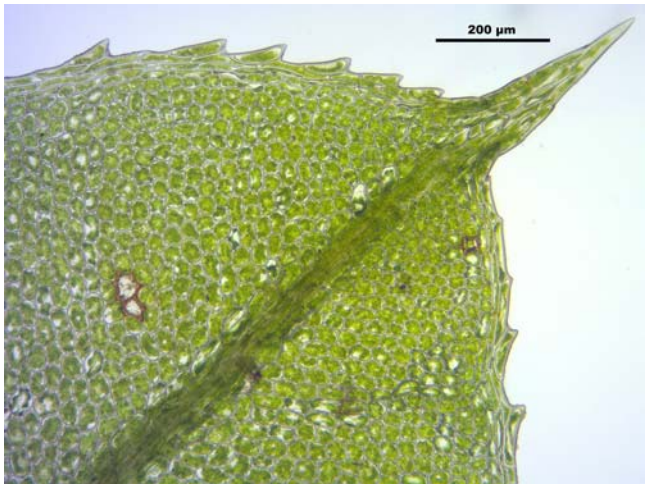

Figure 46. ***Plagiomnium medium*** demonstrating the difficulty in measuring cells in such irregular arrangements. Photo by Hermann Schachner, through Creative Commons.

Leaf Angles

A simple ocular protractor can be constructed using an ocular micrometer (Christy 1987). These instructions are modified only slightly from their publication in The Bryological Times.

1. Using an index card or heavy paper, draw a circle with a diameter equal to that at the top of your microscope eyepiece.
2. Use a protractor and ruler to draw 10 radii in 20° increments in a 90° segment of the circle (Figure 47, **a**).
3. Cut out the circle from (1) and cut a rectangle ~3 x 10 cm from the same card or paper.
4. At the points where the 10° radii meet the edge of the circle, draw corresponding marks along one of the long edges of the rectangle (Figure 47, **b**). Do this along the entire edge of the rectangle to form a scale calibrated in 10° intervals. Discard the circle (or save it for making replacement **collars**).
5. On the rectangle, draw a smaller mark midway between each 10° mark. Midway in the scale, label one of the 10° marks "0," then count in each direction from 0 and mark off 45, 90, 135, and 180°. This makes a scale calibrated in 5° intervals (Figure 47, **c**).
6. Wrap the scale, calibrations on upper edge, around the side of the eyepiece tube, and using adhesive tape, secure the overlapping end to the other end to form a ring of paper around the eyepiece tube (Figure 47, **d**). This is the **collar**, for which the fit should be loose enough that it can be rotated on the tube, but tight enough for friction to hold it at any setting.
7. Put a V-shaped scratch or pencil mark, on the rim of the eyepiece, on a radius parallel to the scale line of the ocular micrometer to serve as an index mark (Figure 47, **e**).

Use of the Ocular Protractor

1. Rotate the eyepiece or move the microscope slide until one of the scale increment lines on the ocular

micrometer is parallel with one side of the angle to be measured. While holding the eyepiece stationary with one hand, rotate the collar with the other hand and set the zero point of the collar at the index mark on the rim of the eyepiece (Figure 47, **f**).

2. While holding the collar stationary with one hand, rotate the rim of the eyepiece with the other hand until the other side of the angle is parallel to one of the scale increment lines on the ocular micrometer (Figure 47, **g**). The degrees of the angle can then be read on the collar at the point directly below the index mark on the rim of the eyepiece.

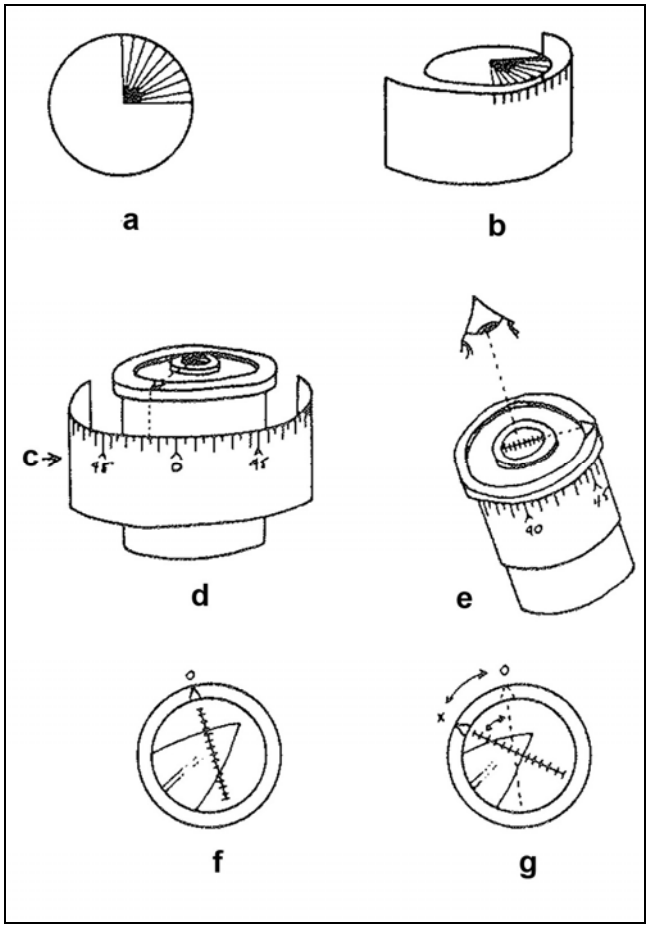

Figure 47. Ocular protractor for measuring angles. Modified from Christy 1987.

Rigidity Index

To calculate the **rigidity index** of **Polytrichaceae** leaves, Sean Edwards (pers. comm. 22 April 2014) multiplies the mean height of leaf **lamellae** (thin sheet of cells standing up along costa of leaf; Figure 48) and divides by the mean width of the **lamina** (expanded portion or blade of leaf; Figure 48), both as mid-leaf number of cells. This permits you to quantify the rigidity of the leaves as a rigidity index. This can be used to characterize different species, even within environmental variation.

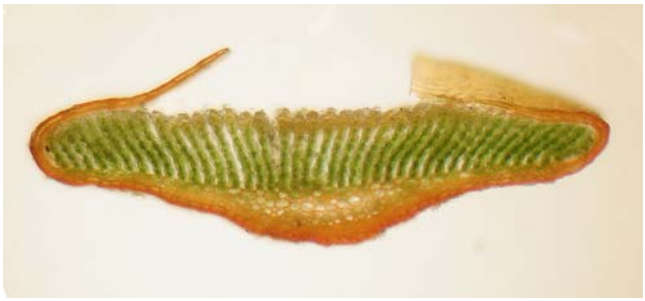

Figure 48. ***Polytrichum juniperinum*** leaf cross section showing vertical lamellae and width of leaf lamina. Photo courtesy of John Hribljan.

Making Drawings

Rod Seppelt (Bryonet 30 December 2022) reports that he was shown a way to use black and white prints to make line drawing images. First, go over the outlines with India ink. Then clear the print in potassium permanganate. This leaves you with the outlines on a white background. But Rod doesn't use this technique because he is able to make excellent drawings by hand.

Older bryologists will remember the camera lucida. This method uses a mirror attached to the ocular of the microscope. Hence, while looking through the microscope you can see the sheet of white paper at the same time and trace around what you see.

David H. Wagner (Bryonet 30 December 2022) has posted a youtube video <https://www.youtube.com/watch?v=pXbVEVnSX4c&t=28s> to demonstrate using photographs to make pen and ind drawings. The method is useful for both macro- and microphotographs.

Summary

Bryophytes have a number of interesting performances, and these can be enhanced by clearing tissues with lactic acid. Capsules can be opened with KOH and NaOCl or heating the slide, depending on the species. To see peristomes under the compound microscope, the capsule should be split. Teeth on whole capsules are best viewed if positioned carefully so they are vertical. Clay can serve as an anchor. Moisture modification can cause the teeth to flex. Spore dispersal can be demonstrated on an agar plate.

Observation of sperm requires a knowledge of the delicate timing. Sperm can be slowed in methyl cellulose and the diaphragm should be adjusted to increase contrast. Other options are DIC microscopy or use of a vital stain such as Sperm VitalStain™.

Adding water to dry leaves can cause leaf movements. placing the base of a plant in dye can demonstrate the movement of solutions in the capillary spaces. Tropisms can be demonstrated on agar plates or other positioning. Etiolation is easily demonstrated in low light.

Dispersal can be demonstrated in splash cups using food coloring. Brownian movement in cells is easily

observed in live leaves. Adding a salt solution to the edge of the coverslip can demonstrate plasmolysis.

Nutrient cycling can be demonstrated by the ability of bryophytes to clear a dye solution in a small cup.

Measurements are important for identification and making descriptions. These will require an ocular micrometer and stage micrometer. These must be calibrated for each microscope and user. Measuring leaf angles may require making your own simple equipment.

Acknowledgments

I thank all the Bryonetters who have shared their excitement and discovery with the rest of us. Please continue to contribute your wonderful stories and demonstrations that excite your students.

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CHAPTER 2-4
LABORATORY TECHNIQUES: PRESERVATION AND PERMANENT MOUNTS

Janice M. Glime and David M. Wagner

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CHAPTER 2-4
LABORATORY TECHNIQUES: PRESERVATION AND PERMANENT MOUNTS

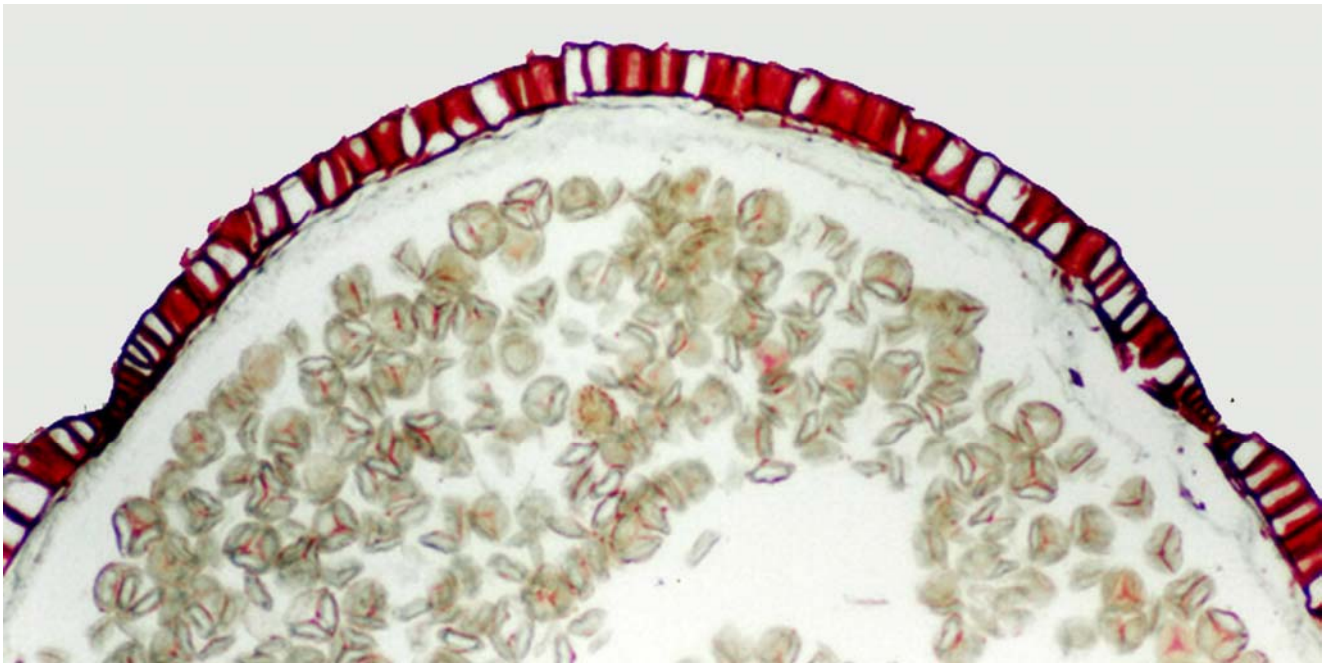


Figure 1. Stained permanent mount of ***Sphagnum*** capsule from Triarch. Photo by Janice Glime.

Permanent and Semi-Permanent Slides: Mounting Media – Mountants

There are lots of considerations in making permanent mounts – ease of use, availability of ingredients, drying time, clearing effect (Angela Newton, Bryonet 16 February 2011). Most people would like their permanent mounts to be durable, surviving being "tossed around." Each mounting medium seems to have its problems, satisfying some, but not all the criteria (Holzinger 1900; Yuncker 1921; Jennings 1935; Iwatsuki 1955; Bowers 1964; Wilberforce 1970; Zander 1983, 1993; Frahm 1990). Nevertheless, as seen in the professionally prepared slide in and available at <http://digitalcommons.mtu.edu/bryophyte-ecology/>., details can become more visible with staining and made to last.

Traditionally, mounting was accomplished with balsam mounts (Jennings 1935) or a synthetic resin like Permount, but slides made with these had to be stored horizontally or the mountant would drift to one slide, carrying the specimen(s) with it. The balsam procedure is time-consuming, requiring dehydration of the specimen in an alcohol series until it is ready to accept an organic solvent such as **xylene** that will mix with the resin. This means that the **mountant** cannot simply be added to a slide once one determines that the mount is suitable for preservation. Furthermore, xylene is highly toxic. MSDS guidelines recommend protection with goggles, respirator, lab coat, and gloves to avoid potential toxicity to "blood, kidneys, liver, mucous membranes, bone marrow, or central nervous system (CNS). Repeated or prolonged exposure to the substance can produce target organs damage."

Des Callaghan (Bryonet 14 December 2018) notes that delicate species with thin cell walls do not preserve well in most mountants. The cell walls collapse in glycerine jelly. A detailed but time-consuming method for mounting delicate liverworts is provided by David Copestake (2015).

Rod Seppelt (Bryonet September 2017) advises making the mountant more viscous by varying the percentages of the glycerol-water mix or the Karo syrup-water mix.

Glycerine to Gum Arabic

Sayre (1941) suggested a gum arabic **mountant** (mounting medium) for bryophytes. At the time, this was a new approach that was superior to glycerine, although Wagner finds that the glycerine mounts of thin sections can

be made overnight and will last for decades if handled carefully. Sayre reports that a combination of glycerine and gum arabic can last for more than a year:

1. Mix 20 g gum arabic in 60 cc distilled water.
2. Let stand covered several hours.
3. Filter through coarse paper.
4. Add 10 cc glycerine and 4 cc formalin (more glycerine may be needed in a dry climate).
5. Place specimen in drop of mix (mountant).
6. Allow to dry for 24 hours in flat position at room temperature.
7. Store flat.
8. Store the mountant in bottle with pipette stopper.

Hoyer's Solution

Hoyer's Solution, also known as gum chloral, was one of the earliest mountants in widespread use for bryophytes (Anderson 1954; Conard & Redfearn 1979; Schofield 1985):

distilled water	50 cc
gum arabic (USP flake)	30 g
chloral hydrate	200 g
glycerine	20 cc

Schofield (1985) recommended allowing the solution to stand for several hours to reduce the number of bubbles; a magnetic mixer can help in this regard as well. Store in air-tight bottles.

Anderson (1954) reported that it was suitable for all mosses he tried (he did not study liverworts) except **Mniaceae** (Figure 3) and ***Tortella*** (Figure 2). In the latter mosses, **Hoyer's solution** caused cell shrinkage and distortion in some species. Lightowlers (1980) expressed frustration at the cell distortion. Anderson did report that both H. L. Blomquist and R. M. Schuster used Hoyer's solution for **liverworts** and that these had held up well, as did ***Sphagnum*** (Figure 4). Schofield (1985) likewise recommended Hoyer's, stating that it results in distortion in leaves of some bryophytes, but it is suitable for most. Hoyer's solution has the added advantage of being an effective clearing agent, so it is helpful for such structures as peristome teeth, capsule exothecial cells, and dense papillae (Anderson 1954). It has the added advantage of not needing **luting** (sealing edges with something like nail polish) (Zander 1993) although unsealed slides will dry out or crystallize much faster than sealed slides.

Figure 2. ***Tortella tortuosa***, a genus in which cell shrinkage occurs in some species when placed in Hoyer's solution. Photo by Des Callaghan.

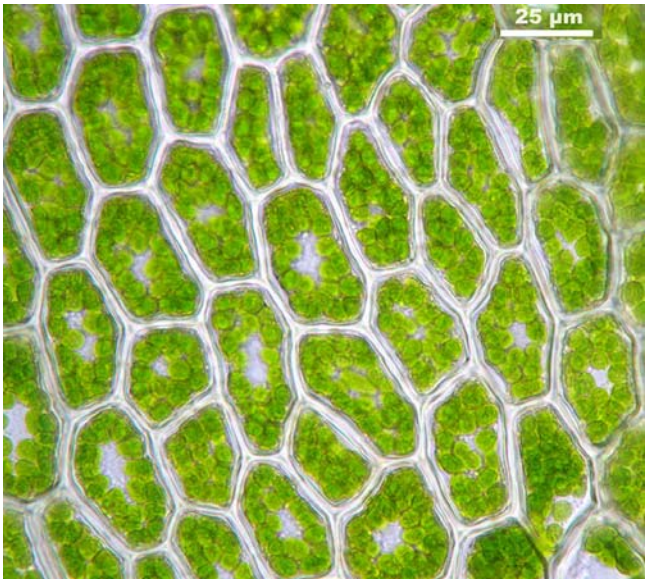


Figure 3. ***Mnium spinosum*** leaf cells. Cells of members if this family (Mniaceae) exhibit cell shrinkage and distortion in Hoyer's solution. Photo by Ralf Wagner <www.dr-ralf-wagner.de>.

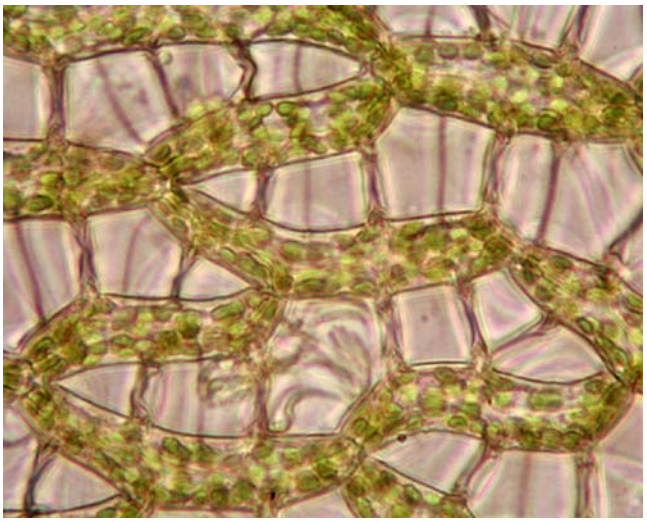


Figure 4. ***Sphagnum palustre*** cells. Cells in **Sphagnaceae** are able to retain their shape in Hoyer's solution. Photo by Malcolm Storey through Creative Commons.

Water Glass Alternative (WGG) for Hoyer's Solution plus Glycerin

This chapter was posted for less than a week when Richard Zander posted this alternative Zander 2013). He touts it as a way to avoid the "tedium of heating slides to melt glycerin jelly." It does not solidify as quickly as glycerin jelly, and its longevity is not known but may be at least as long as a glycerin mount. Even if it crystallizes around the edges, it will still render the bryophyte acceptable.

2 parts water glass solution (sodium silicate solution 40-42 Be)
1 part glycerin (glycerol) mixed with a little water to help it dissolve in the water glass

1. Mix and stir well.

2. Put in capped squeeze bottle or dropper bottle (not a glass-stoppered bottle).
3. Soak specimen in water or 2% KOH solution or water or aerosol solution.
4. If cells are large and thin-walled and apt to collapse, add a drop of pure glycerin and heat to force glycerin into the cells, but this is usually unnecessary even for species with moderately large laminal cells.
5. Add a few drops of WGG over the moist specimen and add cover glass.

In addition to the slow drying time, the basic *p*H may cause some interesting color reactions similar to those when using KOH. On the other hand, its high index of refraction and tinting of leaf cells may make stains unnecessary for ***Sphagnum*** leaves. If stains are needed, they must be basic with this mountant.

Modified Hoyer's for Chromosomes

Concerned about the distortion effects on ***Mnium*** (Figure 3) and ***Tortella*** (Figure 2, Figure 5), Bowers (1964) developed a technique to give better results for making chromosome preparations.

1. First place the material in concentrated HCl, water, and stain (1:3:3).
2. Mix the modified Hoyer's medium:

gum arabic (U.S.P. flake)	20	g
distilled water	60	cc
chloral hydrate	5	g
glycerine	10	cc

3. Mix in above order at room temperature. The solution may need to stand for several hours for bubbles to dissipate. It should be clear and with no precipitate. Store in glass, air-tight bottle.
4. Add stain to Hoyer's medium (1:1) and place drop on slide.
5. Transfer plant material to the medium-stain mixture.
6. Macerate by tapping.
7. Add coverslip.
8. Smear with pressure from a finger.
9. For recently fixed (aceto-alcohol 30:70) or fresh material, use one part aceto-orcein to one part medium. For material stored for a long time, aceto-carmine gives better results.

Figure 5. ***Tortella rigens*** leaf cells, member of a genus in which cells of some species collapse in Hoyer's solution. Photo by Kristian Peters.

Gum Chloral Solution

This solution can serve both as a clearing agent and for making permanent mounts. This recipe has the same ingredients as Hoyer's solution, but in different proportions. The following recipe is from Watson, courtesy of Martin Godfrey:

distilled water	100	ml
gum arabic	40	gm
glycerine	20	ml
chloral hydrate	50	g

1. Dissolve gum arabic first in cold water for 1-2 days.
2. Do not to stir – you will get masses of air bubbles.
3. When dissolved, add glycerine and chloral hydrate and heat until dissolved.
4. Filter hot if necessary.
5. Put drop on a slide and add wet specimen to it.
6. Leave slide horizontal for 2-3 days for gum to set and clear.

Godfrey (Bryonet) reports that gum chloral is great for things like ***Fossombronia*** spores (Figure 6). For structures like perianths, the clearing properties will make the interior structures visible without the need to dissect. The slide can be sealed with a few coats of nail polish to stop the gum from drying out, permitting it to last more than 30 years. Beware, however, that delicate specimens may shrink. If these don't need to be cleared, you can use glycerine jelly, as suggested by Richard Zander. And this actually causes little distortion (Zander 1997).

Figure 6. ***Fossombronia*** spores and elaters. Gum chloral works well as a mountant for these spores. Photo by Tom Thekathyil.

David Long (Bryonet 11 October 2009) reminds us that Hoyer's solution is especially good for liverworts because it can both clear the tissue and preserve it. However, it can cause health issues. Long-term use of **chloral hydrate**, a constituent of Hoyer's solution, can cause addiction, rashes, gastric discomfort, and severe renal, heart, and liver failure (Gelder *et al*. 2005). It should not be used for classroom studies.

Its reputation as a health hazard has made Hoyer's solution difficult to obtain. David Wagner (Bryonet 31 May 2010) tells us that "the primary deterrent to availability of Hoyer's Solution for use as a clearing agent is that it is a controlled substance. In the USA it is a

Schedule IV drug, classed with barbiturates, tranquilizers and sedatives. A prescription is necessary to obtain it. It is/was known by the street name 'Mickey Finn,' a knockout or date rape drug. It has been used (and misused) as a sedative and sleep-inducing agent for over a century and a half; its hypnotic effects seem to be a reason for its abuse as a recreational drug."

A recent product might have some value as a substitute, but its track record is unknown (Villani *et al.* 2013). This product, Visikol™, is made with polychlorinated alcohol and is considered a replacement for chloral hydrate as a clearing agent. It already contains glycerol and works successfully for flowering plant tissues, clearing them in 20-30 minutes, although the authors warn that larger samples may require up to three days. It also works for insects, fungi, and protists, but its usefulness for bryophytes and its effect on bryophyte tissue shrinkage and effectiveness over time remain to be tested.

But it has problems as a mountant as well. It can badly distort specimens by drying them, clear them so much they are nearly invisible, or suffer crystallization (Wagner, Bryonet 31 May 2010). Considering the relative safety issues and undesirable traits of Hoyer's solution, glycerine jelly is a much wiser choice for a mountant.

The distortion problem can be mostly eliminated by soaking the plants thoroughly and adding a little heat to eliminate trapped air (Angela Newton, Bryonet 25 June 1999). Flowers (1973) suggests using a warm coverslip to reduce the bubbles. Then, once the specimen is in the glycerine, it is necessary to wait until the tissues relax before putting on a coverslip (Angela Newton, Bryonet 25 June 1999). An additional problem is the invasion of bubbles from the edges of the coverslip. Interestingly, round coverslips seem to reduce this invasion, but sealing the edges may be a better plan. One can also refill the coverslip during the first few weeks to reduce this problem.

Jonathan Sleath (Bryonet 24 June 1999) agrees with Newton. He has used Hoyer's solution (or gum chloral) successfully for a number of years, following the recipe given in Smith (1978). "The slides have kept well, and could probably be made permanent by sealing the edges of the coverslip with clear nail polish. The material does distort a little at first as water is drawn out by osmosis, but once equilibrium is reached the cell walls seem to regain their normal shape. Of course the cell contents are lost, but the clearing properties of the solution can be quite useful." Despite all the concern over health issues, Sleath reports that chloral hydrate, the major constituent of Hoyer's solution, is still occasionally used in pediatric practice in the United Kingdom because it is so safe. Chloral hydrate is not a controlled substance in the United Kingdom at this time (Wikipedia, 2013). When he makes a permanent mount, he seals the coverslip on all four sides, unlike the corner technique of Wagner.

Allan Fife (Bryonet 24 June 1999) suggested that soaking dissected bryophyte tissue overnight in **90% lactic acid** will largely prevent the cell wall collapse that is a common feature of Hoyer's mounts, particularly of thinner-walled cells. But, that does not remove its dangers from its chloral hydrate. The Oxford MSDS gives the ORL-RAT LD50 480 mg kg^{-1}. (ORL=Oral, RAT=rat, LD50=the dosage that killed 50% of the rats). So, if you weigh 70 kg, you would need to ingest 33.6 grams of choral hydrate to have a 50% probability of death (assuming you were a rat). According to Anderson's recipe there are 0.2 grams of chloral hydrate per ml of Hoyer's Solution. Therefore if you imbibed ca. 160 ml of Hoyer's Solution you would have a high likelihood of not seeing the next sunrise. The Oxford MSDS recommends safety glasses and ventilation when handling chloral hydrate. It is listed as a skin, eye, and respiratory irritant. You should also wear gloves and take care when using Hoyer's, and make a point of rinsing your hands after using it.

Belen Albertos (Bryonet 25 June 1999) reported that his lab uses **Kaiser's glycerol gelatin** to make permanent slides for microscopy. This is available in Europe, Asia, South America, and Australia from Merck, ready to use. Interestingly, it is not available through this supplier in Canada or the United States, although it is available in Mexico. To use it, you need to warm a portion of the solution to about 40°C. Once your slide is prepared as a water mount, place a drop of the warm solution on the edge of the coverslip. If there is excess liquid, pull the glycerol under the coverslip by placing the edge of a paper towel on the opposite edge of the coverslip. Albertos suggests placing the slide on a slope to let the glycerine run down and displace the water, but the paper towel method is probably the most effective. When solution completely covers the material, place the slide on a level surface to cool. The coverslip will keep the specimen in position during storage, but there might be a slight loss of color. Albertos reports that slides made in this way are still in good condition after five years. However, he warned that a colleague found that liverworts get altered quickly.

Lightowlers (1981) suggested a modification of gum chloral to preserve the shape of cells:

1. Presoak the specimen in an aqueous solution of 50% polyethylene glycol (PEG) 400 grade for about 12 hours (until thoroughly penetrated). 50-100% glycerol or 100% lactic acid are somewhat less effective.
2. Remove from solution after soaking and blot thoroughly with tissue paper. Too much PEG reacts with gum arabic to make an opaque precipitate. Too much glycerol slows the setting time. Excess lactic acid crystallizes upon drying.
3. Place specimen in drop of mountant on slide & dissect as needed.
4. Tease out bubbles and cover with coverslip.

Glycerine, Glycerol, and Glycerine Jelly

Hoyer's solution has been used for many decades as a mountant, but its toxicity (chloral hydrate) has decreased its availability and popularity. Furthermore, with Hoyer's solution, slides remain sticky and are easily smeared. Glycerine seems to be a viable alternative.

Glycerol is made from glycerine (=glycerin). Glycerine is the solid state, whereas glycerol is the liquid state. The formula is the same. Several Bryonetters have recommended this alternative (Ida Bruggeman, Cyndy Galloway, Martin Godfrey, Paul Davison, David Wagner, Richard Zander).

In 1900(!), Holzinger suggested mounting bryophytes in **glycerine jelly**. He improved upon the method of using mounts in jelly between mica sheets (replaced now by

coverslips) by using two thicknesses of paper – one ordinary writing paper, one cardboard that is slightly heavier than postal card paper (poster paper?).

1. Cut these into strips the length of a microscope slide and slightly wider.
2. Fasten at one end with paste to keep them from slipping.
3. Lay glycerine jelly mount in center of paper slide.
4. Cut through both thicknesses with sharp pointed pen-knife.
5. Cover inside of cardboard with paste.
6. Lay jelly mountant down over it.
7. Press down the thin paper, being careful not to misplace anything.
8. The two thicknesses of paper form a frame for the mount with margin of white paper to put data about the specimen.

Ida Bruggeman, Bryonet, reports that specimens in Hoyer's mounting medium will dry out after a while, losing contrast and making many details invisible. In comparison, many of her glycerine gelatin slides, sealed with nail polish, have lasted more than ten years, but they, too, dry out or get fungal infections after 15-30 years.

Glycerine appears to be the simplest and safest method of making permanent slides. Once you are through observing anything that might move and are ready to make it permanent, you can simply add a drop of glycerine at the edge of the coverslip (David Wagner, Bryonet 12 February 2001). It can be drawn under the coverslip by placing a bit of paper towel on the opposite edge of the coverslip and letting capillary action pull the excess water away. If the slide is left in the open for several days, water will evaporate and the glycerine will penetrate the specimen. Once the specimen is impregnated with glycerine, affix the coverslip by placing a drop of clear nail polish at the corners of the coverslip. Wagner recommends NOT trying to seal all the sides with nail polish because they seem to leak, but the ones affixed at the corners can last 20 years or more. Centering the coverslip will minimize the leakage of glycerine from the slide. You can clean the coverslip when needed on this more-or-less permanent mount by using alcohol on a cotton swab. Unfortunately, the oil bodies will not preserve.

It takes practice to determine how much glycerine to use. The needed amount varies with the thickness of the specimen, with a thin mount of leaf sections typically requiring only a single drop. More glycerine can be added, but as the slide dries, air may be drawn under the coverslip, trapping bubbles when more is added. If you add too much, you can blot it with thin strips (5 x 20 mm) of tissue paper, toilet paper, or paper towel. This process should be repeated until the glycerine barely starts to draw back from the edge of the coverslip.

Some bryophytes will shrink and curl when the glycerine is added. This is an indication that the change has been too quick. Dilute the glycerine and add it more slowly, allowing some of the liquid to evaporate, then adding more.

Glycerine quickly follows capillary spaces. While this is an advantage for drawing it under the coverslip, it is likewise a danger for drawing it out. If the slide contacts another slide next to the coverslip, the glycerine will creep to the edge of the slide, then follow the capillary spaces between the slides, ultimately travelling in the capillary spaces under the slides. Within a few days to weeks, all the glycerine will have followed this capillary route and will vacate the coverslip space. To help prevent this, be sure the coverslip is perfectly centered on the slide before you add nail polish. Wagner finds that it is best to add the nail polish right after the glycerine has been applied and before it is set aside for drying.

In 1997, Zander retracted his earlier advice (Zander 1983) on the use of lactophenol gel made with methyl cellulose. While this seemed to be a good alternative for clearing without collapsing the cell wall structure, making nice mounts, it dried out within six months, making the mounts unusable (Zander, pers. comm. 19 July 2012). Zander (2007) contends that the perfect mounting medium still does not exist, despite the many presented by past bryologists (Davis 1909; Sayre 1941; Anderson 1954; Bowers 1964; Wilberforce 1970; Lightowlers 1981; Zander 1983; Frahm 1990). He (Zander 1997, 2007) recommends mounting in **glycerine jelly** (= a mixture of glycerine and gelatin that is used in histology for mounting specimens). This glycerine jelly medium sets fast, is safe for delicate tissues, preserves the color responses of cell walls to potassium hydroxide, has a high index of refraction, has a long life, and the ingredients are safe and inexpensive. Its slow evaporation rate makes it virtually permanent with **luting** (sealing with a paste, nail polish, or other sealant around the coverslip).

Glycerine jelly requires a longer procedure because it must be melted before it can be used. But melting degrades the gelatin so it does not set, so it must be melted just before it is used. If the slide gets too hot, it will destroy delicate organs such as axillary hairs. Coloration from KOH tests will disappear in a few days. And, the glycerine will evaporate over a period of years. Evaporation can be retarded by storing the slides in a closed container, but that presents an additional nuisance.

Glycerine Jelly Preparation (Zander 2003)

Richard Zander (2003) has suggested using glycerine jelly instead.

1. Take 2 packets (7 g each) of gelatin (Knox brand works fine).
2. Mix in 50 ml cold water to hydrate.
3. Heat but don't boil, while still stirring, until the liquid is clear or at least there is no undissolved gelatin.
4. Swirl it to dissolve all gelatin.
5. Add glycerine to make 200 ml.
6. [Optional: Add a crystal of thymol to keep down bacteria and fungi.]
7. Heat for about an hour until everything dissolves and the liquid is clear.
8. Pour on a clean PVC (polyvinyl chloride) pan with a flat bottom to make a thin layer.
9. Leave uncovered or cover with cloth to prevent dust overnight to several days to allow most of the water to evaporate.
10. Peel off the thin, flat sheet of glycerine jelly.
11. Roll into a kind of jelly roll.
12. Slice the roll crosswise into neat, tight curlicues or helices about 0.5 cm wide.

13. Store in a plastic box.
14. Pinch off a small piece when wanted.
15. If there are bubbles, reheat in a beaker (water bath is helpful) and let stand as liquid. Do not keep hot for a very long time, as heat denatures the gelatin.
16. The glycerine jelly will be hard to remove from the plate unless the water portion has evaporated. It is best to evaporate the water portion with heat since glycerine absorbs water, to some extent, from the air.

Using Glycerine Jelly

1. Place bryophyte material in a drop of water on a slide and soak it.
2. If the water is not absorbed readily, heat the slide slightly with a butane cigarette lighter, or start with warmed (not boiling) water.
3. Make desired sections and arrange the material on the slide.
4. Pinch off a bit of the glycerine jelly and place on slide and heat the slide evenly. (Heating one spot can break the slide.)
5. Arrange the material and add a coverslip.
6. The jelly hardens in a minute and may be mailed after cooling.
7. If clearing is needed, first dip the moist plant in lactic acid for a minute or so, or heat in pure lactic acid before preparing the glycerine jelly mount.
8. The jelly can be kept liquid for a short time on a hot plate, but prolonged heat turns the jelly brown and the gelatin breaks down so that it won't harden.
9. NOTE: Glycerine will eventually dissolve calcium carbonate.

Making Semi-permanent Mount

1. From wet mount, blot specimen to remove excess water.
2. Add small drop or drops of glycerol to slide with specimen.
3. Heat with lighter under slide till boiling (this pumps up the tissues after an initial collapse).
4. Do dissections if needed.
5. Add piece of glycerine jelly and heat until melted (try to avoid boiling a second time).
6. [Try transferring boiled specimen atop solid piece of glycerine jelly on clean slide, then heat just to melting; this helps minimize bubbles and helps minimize mess.]
7. Arrange specimen quickly and if needed cover with a coverslip.
8. Add label on left side and store slide flat in air-tight box.

Dave Wagner has been mounting liverworts on his slides in just glycerin for years, and seems to have much success. However, Des Callaghan (Bryonet 25 November 2013) found that some bryophyte species, such as ***Thuidium*** (Figure 7), do not reach "full turgor" and remain somewhat dehydrated in glycerol plus alcohol (the alcohol evaporates, leaving the glycerol).

Richard Zander (Bryonet 6 December 2013) suggests heating the slide of water-mount cells in added glycerol on a hot plate or coffee cup warmer. Cells of such sensitive species as ***Tortula hoppeana*** (Figure 8) expand to full turgor and stay that way. He also recommends using a 1 to 1 mixture of glycerol and Elmer's Clear School Glue (apparently a thick polyvinyl alcohol solution). The index of refraction remains high. When the water in the glue is evaporated it makes a semi-solid mount. If the cells collapse, heat on a hot plate or cup warmer as with pure glycerol. Make sure the glue and glycerol are well mixed or the slide will seem to "weep" glycerol.

Figure 7. ***Thuidium delicatulum***, a species that does not rehydrate well in glycerol plus alcohol. Photo by Michael Lüth, with permission.

Figure 8. ***Tortula hoppeana***, a sensitive species that expands well in water plus glycerol with heat. Photo by Paul Wilson, with permission.

Clearing

If you need a more transparent specimen, you can clear it first by using a mixture (1:1) of glycerol and lactic acid [see step 7 in "Using Glycerine Jelly" above (Zander 1997)]. You can make mounts more quickly by adding glycerine jelly directly to a blotted wet mount and heating to boiling (bubbles may be troublesome). Dip the moist plant in lactic acid for a minute or so (or heat in the pure acid) before preparing the glycerine jelly mount.

Alternatively, you can keep a small hot plate near your microscope. Set a microscope slide with the wetted plant (or sectioned material) in a little water (or a mixture of water and a little glycerine) and a cube of glycerine jelly on the hot plate. After a moment or two, the glycerine jelly melts and some of the extra water evaporates. Remove the slide, arrange the material, and put a coverslip on it. The hot plate can be one of those coffee warmers you can pick up in a flea market cheaply. This eliminates heating a cube of glycerine jelly on a slide, which can break the slide if it is not heated evenly with the cigarette lighter or other point-source heat source. You can rig it so that the hot plate warms up whenever the microscope light is on.

Zander (1997) adds that glycerine will eventually dissolve calcium carbonate. Also, if you make the glycerine jelly with a high concentration of gelatin, then if you work on your specimen in a water/glycerine mixture (which slows sections from flying around when you make them), any added glycerine jelly will not be too dilute (when mixed with the pure glycerine on the slide) to harden.

For **Mniaceae** (Figure 9) and other large-celled bryophytes, you may need modifications. Richard Zander (Bryonet 9 November 2009) suggests that you strip leaves in water or Pohlstoffe solution or 2% KOH, then add 2 tiny drops of pure glycerine to the water or solution mount. Make cross sections and arrange leaves nicely in the unmixed water/glycerine mount, add a fingernail-sized clod of glycerine jelly, heat with one of those butane lighters with the nice torch flame, taking care to heat the slide fairly evenly so it does not break. Heat until the glycerine jelly just melts, or before it is completely melted, rearrange the leaves and whatever else is on the slide before placing a coverslip on it. After it cools it should be solid and ready to mail or bang around the lab without drying. Don't use 2% KOH with liverworts as the leaf cell walls are attacked.

Figure 9. ***Plagiomnium cuspidatum*** dry, a moss that repels water instead of absorbing it. Photo from Dale A. Zimmerman Herbarium, Western New Mexico University.

Dan Marsh (Bryonet) similarly suggests Frahm's (1990) Solution, 1:1:1 glycerine, water, mucilage (the brown glue-type from school supply sections of stores). For study of variation in ***Sphaerocarpos*** spores (Figure 10), this solution served not only as a satisfactory mounting medium but also cleared the spores quite nicely.

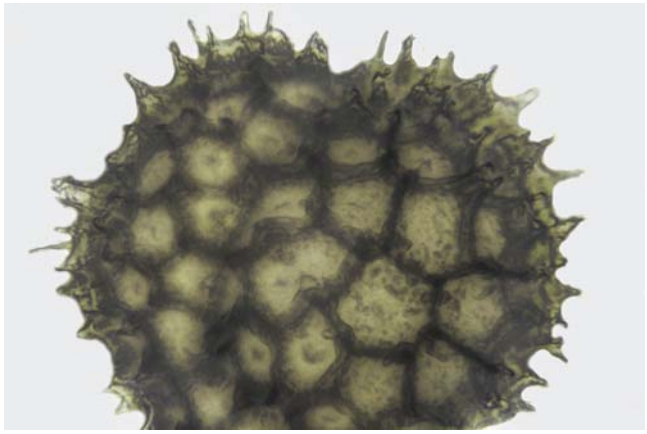


Figure 10. ***Sphaerocarpos michelii*** spore, a structure that clears well in Frahm's solution of glycerine, water, and mucilage in equal parts. Photo by Dick Haaksma, with permission.

DMHF (5,5-dimethyl Hydantoin Formaldehyde)

In his search for a better mountant, Zander (2007) uncovered 5,5-dimethyl hydantoin formaldehyde (DMHF), discussed by Steedman (1958). It is a water-soluble resin generally used in such products as cosmetics, adhesives, coatings, inks, and textiles. Although a similar chemical is used to gradually release the carcinogenic formaldehyde, DMHF releases little or none. Zander found the solution easy to use with tested species of ***Tortula*** (Figure 11) and ***Mnium*** (Figure 3) but, when hardened, the mountant resulted in somewhat collapsed laminal cells and a low index of refraction. Although DMHF would indeed make permanent mounts of bryophytes that are less collapsed than, *e.g.*, acrylic solutions, the low index of refraction makes it difficult to examine anatomical details such as laminal papillae. When mixed with glycerine, visibility improves but the material will not set well.

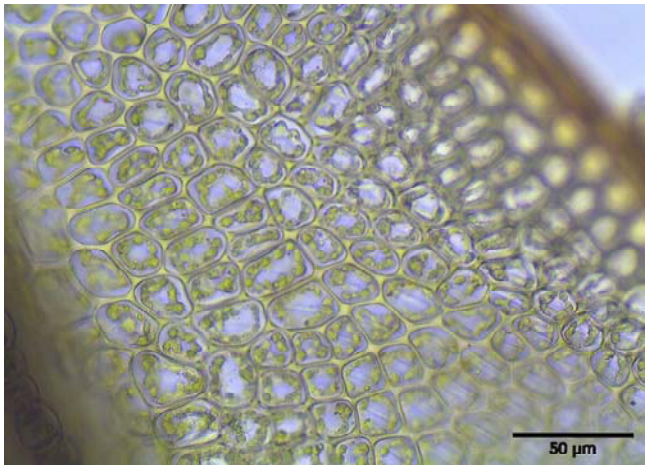


Figure 11. ***Tortula subulata*** leaf lamina cells, member of a genus wherein mountants may collapse the laminal cells. Photo by Kristian Peters.

PVA

Zander (2007) credits Howard Webb with alerting him to polyvinyl alcohol with glycerol (PVAG) and polyvinyl alcohol with glycerol and borax (PVAGB) media (Salmon 1954; Dioni 2007; Webb 2007). However, Anderson (1954) cautions against use of PVA (polyvinyl alcohol) as a mountant because after a few months the plants begin to shrink and distort, ultimately becoming unrecognizable. The addition of glycerol and borax had the potential to prevent those problems.

If in fact PVA is an acceptable medium, it is easy to obtain. It is the primary ingredient of transparent glues and glue gels, and one could experiment with these as well. Carolina Biological Supply has a powdered form that can be made into a thick syrup by heating it for a long time in water. Zander points out that when used alone the PVA causes the same problems as in Anderson's warning, those also known for acrylic resins and DMHF, where cell walls collapse and the index of refraction is low. Zander reports that the addition of glycerine gives the mountant a high index of refraction, but the resulting hardened medium is cloudy. The addition of borax does not help, nor does decreasing the water. He couldn't recommend it.

Elmer's glue consists of PVA, water, ethanol, and acetate. It can serve as a mounting medium, as shown by David Meagher (Figure 12).

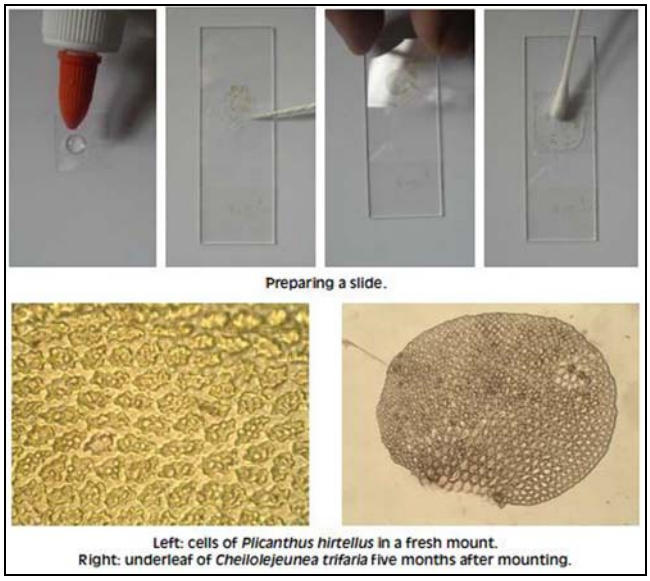


Figure 12. Meagher permanent mounting methods using glue. Photos by David Meagher, with permission.

Karo Syrup

A cheap and readily available mountant is Karo Syrup. This is the mountant of choice at the State Herbarium of South Australia. Graham Bell (pers. comm.) reports that it works better than other media in that dry climate. He adds phenol (2-3 %) to the dilute Karo mix to prevent fungal and bacterial contaminants. Standard dilutions of the Karo syrup are 20, 50, and 80%. It is often necessary to start with a lower concentration, let the slide dry for a day or two, then backfill it with more Karo solution at a higher concentration. Some of the slides made in this manner are more than 70 years old and still useable. Some stains seem to present problems, but once the slides stabilize they remain in good condition.

Polyvinyl Lactophenol

This mountant has the advantage that it need not be luted (Zander 1993) because it actually sets, which Hoyer's does not (Fisk 1991). However, cells collapse in it. Zander (1983b) suggests instead a lactophenol gel because it usually does not collapse the cells. It also has a high index of refraction. However, Rod Seppelt (Bryonet 12 February 2001) warns that heating solutions with **phenol** needs to be done with care. The MSDS data sheet considers it to be very hazardous when in contact with skin or eyes and dangerous if ingested or inhaled. It can cause corneal damage or blindness if it contacts the eye and can cause blistering of the skin. Severe over-exposure can cause lung damage, choking, unconsciousness, or even death. Use a hood if available and avoid breathing the fumes:

lactic acid (=2-hydroxypropanoic acid)	20 cc
phenol, crystal (=carbolic acid)	15 g
distilled water	15 cc
methyl cellulose powder (=cellulose methyl ether, of viscosity 25 cP in 2% solution of lowest viscosity available)	6 g
ethylene glycol (=1,2-ethanediol)	35 cc

Procedure:

1. Mix phenol with lactic acid, dissolving with gentle heat.
2. Add water and stir.
3. Heat to just boiling (**use fume hood**).
4. Add methyl cellulose powder and stir vigorously into hot solution to dissolve (reheat if necessary).
5. Add ethylene glycol last.
6. Pour into glass cylinder and let stand to allow bubbles and undissolved material to rise.
7. Allow to settle for a day or two, then remove any floating particles.
8. Pour clear liquid into storage bottle.
9. Use small bottle with applicator wand in lid or plastic squeeze bottle with fairly wide opening (4 mm) to place drop or two on slide.
10. If specimen is incrusted with carbonates, first soak them in drop or two of dilute HCl to prevent bubble formation in lactophenol gel.
11. Specimens previously soaked in KOH should be neutralized with drop or two of dilute HCl before mounting in lactophenol gel.
12. Moist plants may form a precipitate in the lactophenol gel, but it will dissolve with stirring.
13. Arrange the plants and leaves on a **clean** slide while the gel is still liquid.
14. Add coverslip to preserve for 1-2 months.
15. For semi-permanent slides, it is best to seal the mount with a **lutant** like clear fingernail polish. Nail polish does not adhere well to glass, so varnish might be substituted. Some bryologists (*e.g.* Watson 1963) prefer **circular coverslips** because their lack of corners makes them adhere better.

16. For a more permanent lutant you can use one of the following:
    poly (ethyl methacrylate) with butyl benzyl phthalate as plasticizer (Krystalon®Harleco, Gibbstown, NY 08027 USA) (an artificial balsam)
    polyurethane gloss finish (used for wood floors) – keep slide out of light
17. Apply lutants liberally to make a good seal, but avoid having a ridge that might interfere with changing objectives on the microscope.
18. Keep lutants in a balsam bottle or small disposable applicator bottle; keep more than half full to help exclude air.
19. Keep disposable wipers on hand to keep tools and hands clean from the reagents.
20. A fan may be needed to carry away vapors.

Aquamount Improved

Matt von Konrat (Bryonet 25 June 1999) suggests Aquamount Improved, from BDH Laboratory, an improvement over Aquamount that contained phenol. This solution comes ready to use and is relatively cheap. It avoids the problems of tissue shrinkage.

Figure 13. Kleermount, sold in 100 ml bottles from Carolina Biological Supply Co. Photo by Carolina Biological Supply Co.

Kleermount, Xylene solution #2

Kleermount (Figure 13), suggested by Volkmann-Kohlmeyer and Kohlmeyer (Kohlmeyer & Kohlmeyer 1972; Volkmann-Kohlmeyer 1996) for fungi, works well for bryophytes as well (Martin Wigginton, Bryonet). Kleermount is available from Carolina Biological Supply Company, 2700 York Road, Burlington, NC 27215-3398. International orders: (+1) 910-584-0381; FAX (+1) 910-584-3399). They provide a Material Safety Data Sheet (MSDS) for the solution, which warns that it is harmful by inhalation, possibly causing irritation to the respiratory tract. Prolonged exposure may result in an allergic reaction; it can cause eye and skin irritation. If ingested it may cause gastrointestinal discomfort. It is also a fire hazard: closed containers of Kleermount Xylene solution exposed to heat may explode. In short, its use requires caution and common sense.

Serge Hoste (Bryonet 24 June 1999) adds that Kleermount causes serious plasmolysis in ***Plagiomnium*** (Figure 14), ***Funaria*** (Figure 15), ***Amblystegium*** (Figure 16), and others. He advocates "using a medium with the highest water content possible. A gelatine-glycerol-water and PVA-glycerol-water, with a few drops of thymol added as fungicide, is claimed to keep for more than 20 years and conserve color to a much larger extent than with the use of chloral hydrate. Good sealing around the edges of the cover slide is paramount but can easily be obtained by applying two (or more) generous coatings of clear nail varnish around the edges of the cover glass."

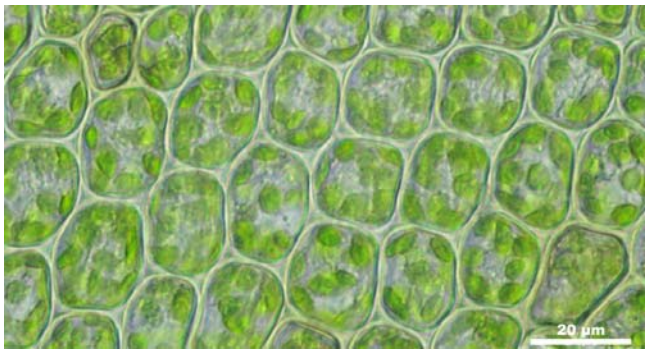


Figure 14. ***Plagiomnium undulatum*** leaf cells. This is a member of a genus in which leaf cells plasmolyze in Kleermount. Photo by Ralf Wagner <www.dr-ralf-wagner.de>.

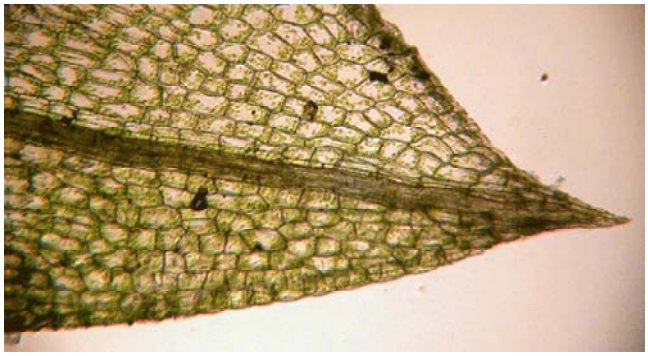


Figure 15. ***Funaria hygrometrica*** leaf cells. This is a member of a genus in which leaf cells plasmolyze in Kleermount. Photo by Bob Klips.

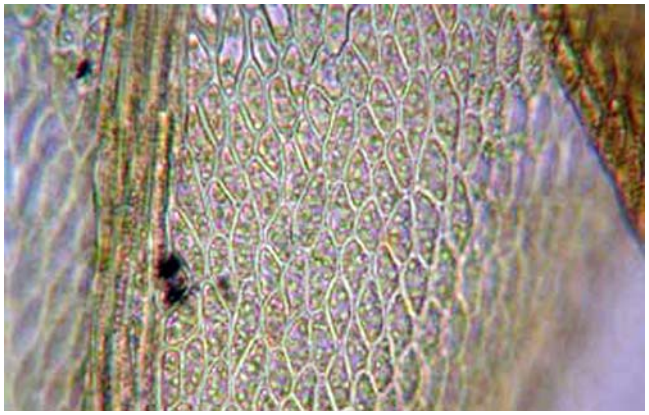


Figure 16. ***Amblystegium varium*** leaf cells. This is a member of a genus in which leaf cells plasmolyze in Kleermount. Photo by Bob Klips.

Fluoromount-G

Bernard Goffinet (Bryonet 12 February 2012) recommends Fluoromount-G. This mountant is available from Fisher (OB100-01, 25 ml for 25$ before discount, good for "500 slides"). It is toxic and should be handled with care. The MSDS data sheet states that it is potentially harmful if ingested and warns "Do not get on skin, in eyes, or on clothing. Potential skin and eye irritant." It has the advantage that it does not bleach the leaves, nor alter them in any way (at least within the two weeks following its use). It is water soluble, so it can be used for mounting wet specimens.

Gray-Wess Mountant

Uwe Schwarz (Bryonet 12 February 2012) suggests Gray-Wess as an alternative mountant:

polyvinylalcohol	2 g
glycerine	5 ml
lactic acid	5 ml
water	10 ml

Mix everything together and heat it in a test tube in hot water until the liquid becomes clear. If the specimen has calcareous incrustations, you should skip the lactic acid because it will cause a lot of bubbles.

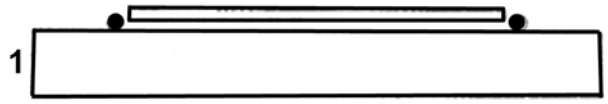
Double-Coverslip Method

Martin Wigginton (Bryonet) suggested the double-coverslip method (Figure 18), published for fungi (Figure 17) by Volkmann-Kohlmeyer and Kohlmeyer (1972; 1996), but originally introduced by Diehl in 1929. Although this method was developed for preserving fungal spores, it works well for bryophytes. The method is more time-consuming than just using fingernail polish, but the results are much more permanent. This method should be used by anybody who needs to prepare voucher specimens, and it should always be used for preserving type material where slides are needed.

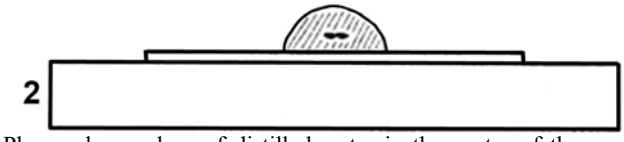
Figure 17. Ascospores of a fungus, using a double coverslip, with appendages perfectly preserved after 29 years. Photographed using Nomarski interference contrast and quartz filter. Photo from-Kohlmeyer & Kohlmeyer 1996.

Tocci noted that shellac was used in the 1800's and lasts about 100 years. One can also use Paraloid B-72 in acetone. But she also recommends the double cover slip method that is popular for mounting fungi (Kohlmeyer & Kohlmeyer 1972), and that can be used with glycerine. It permits observation of living material that can subsequently be made into a permanent mount.

Double Coverslip Method of Kohlmeyer and Kohlmeyer:



Place a 25 x 25 mm coverslip on a clean 76 x 26 mm glass slide, sealed to it with two drops of distilled water.

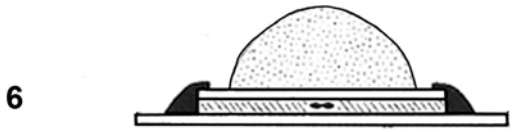


Place a larger drop of distilled water in the center of the cover glass. Add the specimen to the drop.

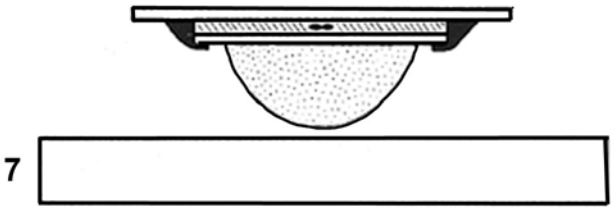
Use the smaller coverslip (18 x 18 mm) to cover the drop with the specimen, permitting immediate viewing of the living material with a compound microscope. Immersion oil can be used if necessary, but it is messy and must be cleaned off, endangering what has been accomplished. It is better to save it until the slide has been sealed and dried.

After making all the observations, measurements, and photographs you need for now, add a droplet of concentrated glycerine to the water at one or two sides of the small coverslip to prevent drying out. The slide must be stored horizontally in a dustproof container for a few days to allow the water to evaporate. Excessive water plus glycerine can be removed from the edges of the larger coverslip easily with filter paper – or if needed, add more glycerine.

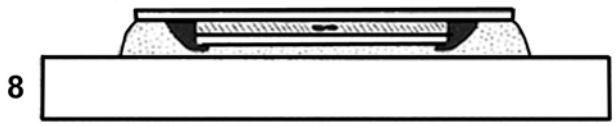
Seal the mount with a thin ring of clear fingernail polish. It is best to repeat this step after an hour to be sure the glycerine is perfectly sealed.



When the nail polish is fully dried, remove the large cover glass from the slide using a needle. Place a drop of mounting medium on the small cover glass. It will take some experimentation to get the right amount.



Turn over the preparation and place it on the slide, gluing it to the slide.



The drop of mounting medium flattens out, but this will happen more readily if a small weight is put on top, *e.g.* some of the larger nuts from a nut and bolt set. These are small, so a supply can easily be kept in the lab. The medium will ooze out and surround the edges of the small cover glass, permanently sealing in the small cover glass and nail polish. Any excess medium squeezed out at the sides can be taken off with a needle syringe. The preparation must be stored horizontally until the medium is hardened, but it can be used after a day, should further microscopy be necessary. The sealing procedures are best done under a hood to avoid breathing the toxic fumes of the medium.

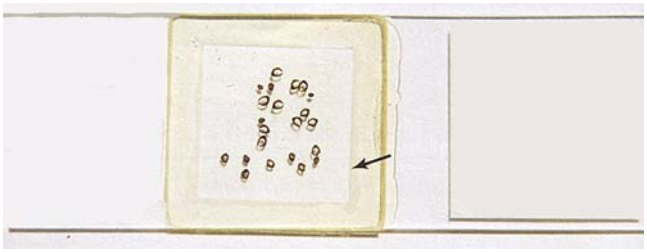


Figure 18. Double-coverslip slide showing smaller top coverslip (arrow) and nail polish seal. Note the blank label on the right. Paper labels can easily come off, so using a slide with frosted glass on one end may be a preferred solution. Labelling should be done with waterproof ink and a Rapidograph pen. Photo modified from Volkmann-Kohlmeyer and Kohlmeyer 1996.

Since this slide and specimen can be used again and again, it is wise to check it for visual clarity of important structures before beginning the sealing process. Be sure that both sides of the leaf can be seen, that at least one leaf base can be seen clearly, and that papillae, if present, can be seen. The latter often requires showing a bent surface of a leaf. Once the process is complete and thoroughly dry, the slide can be placed in a minipacket for protection and stored in the packet with the specimen. This will serve the added advantage of preserving more of the specimen because it won't be necessary to use more material to make more slides.

The method requires two coverslips of different sizes, a large one (25 x 25 mm) and a smaller one (18 x 18 mm). The large coverslip is attached to a clean slide (76 x 26 mm) by using two droplets of distilled water (to avoid mineral deposits). A somewhat larger drop of water is placed on the coverslip and the specimen added. The smaller coverslip is then used to cover the specimen and water.

This method seems to lack a preservative, so one might want to follow the advice of Norbert Stapper (Bryonet 13 February 2001) and add camphor or phenol. We don't know their long-term effects on the slides, but see the warnings of health risks of phenol discussed above.

Lutants – Sealing Slides

Once the mounting medium is reasonably dry, it is usually necessary to seal the coverslip (Diehl 1929). The medium remains somewhat fluid, so the coverslip is essentially floating. David Wagner (Bryonet 31 May 2010) recommends painting with a **lutant** of clear fingernail polish on the two sides of the coverslip parallel with the length of the slides. He cautions against enclosing the coverslip completely because it may become necessary to add more glycerine later. Glycerine at the edges may prevent the nail polish from sticking, in which case the slide may need to be cleaned. Excess liquid should be drawn off first with a paper towel at the edge of the coverslip. You should finish the cleaning of the remaining residue with alcohol or a wetting agent on a cotton swab. It is a good idea to check to be sure that the specimen(s) remains in a suitable position before affixing it permanently. The polish should be spread well away from the edge of the coverslip to give the polish more adherence outside the influence of the glycerine.

In addition to keeping the specimen moist, a coverslip that is tacked down is much easier to clean. Dust will easily accumulate over time and glycerine may invade the surface from a neighboring slide. Wagner advises cleaning lengthwise with a cotton swab moistened with alcohol, being careful to avoid the open edges of the mount so the alcohol is not drawn under the coverslip.

Nail polish has been criticized as not being permanent, but permanent slides need to be sealed with a **lutant** (sealer). Richard Zander (Bryonet 12 December 2018) recommends a 70:30 mixture of clear glue (*e.g.* Elmer's) and glycerin. The glue is a polyvinyl alcohol and lasts a long time. Since lutants usually don't last more than a decade, it is necessary to make sure you can easily soak the slide and remount the specimen. The polyvinyl alcohol is suitable for this purpose because it is soluble in water.

But David Wagner argues that Sally Hansen's "Hard as Nails" clear fingernail polish will last more than 40 years as a lutant, with pure glycerine as a **mountant**

(mounting medium). Instead of ringing the cover glass, he uses it to tack the cover slip in place. This permits the cover slips to be cleared of dust in later years without disturbing the mount. The mountant of glycerine is placed at the edge of the water mount over night so that the glycerine can slowly replace the water. Once the mountant has permeated the mount, the cover glass can be tacked in place with the nail polish.

Reviving Dried Slides

All is not lost if your slides dry out. Glycerine mounts can be revitalized by adding water with a good wetting agent around the open edges of the coverslip (David Wagner, Bryonet 31 May 2010). This will gradually loosen the coverslip so it can be removed carefully with your needle-point forceps. Nail polish usually comes off with the coverslip and can easily be broken off. Polish left on the slide can be scraped away. Make sure the material you need is still on the slide before placing a new coverslip. Otherwise, if important specimens are stuck to the removed coverslip, it is best to re-use that coverslip. Flood the surface of the mount area with your wetting agent mix and gently lower the coverslip as you would with a fresh mount. Add glycerine to the edge of the coverslip and let it sit to dry and infiltrate the specimen. Continue processing it as you would a new mount.

Cleaning Slides

Des Callaghan (Bryonet 11 December 2013) has a great way to clean your dirty slides, whether for re-use or permanent slides that have become smeared. Rub them with a paste-type cleaner between your fingertips. Callaghan uses the UK brand Astonish; a tub will last a lifetime. The paste washes off easily and leaves the glass surface spotless.

Labels

Stick-on labels are available, but these eventually come off. A more permanent solution is to use a slide with an etched area for writing (Chris Cargill, Bryonet 11 December 2013). There is nothing to come off, and Cargill says that permanent markers do not wash off with water and if stored in the dark do not fade appreciably. Non-permanent ink does come off. One can also use a diamond-tipped pen to inscribe the slide permanently. This can be done with an accession number, but the accession list could get misplaced, so it is better to put all information on the slide.

David Wagner (Bryonet 10 December 2013) has used Scotch brand Magic Mending Tape for years, long enough to consider it of archival quality (good for at least a couple of decades). It is quick, easy, and reversible (Figure 19):

1. Strip off a 2 inch (5 cm) length of 3/4 inch (~2 cm) wide tape and tape the slide to the work bench. This holds the slide tightly in place so that writing very small is possible.
2. Write specimen information on the tape. I always use a pencil because there's no wait for ink to dry. Erasable, too. This is the stage to add glycerine to the edges of the coverslip for a semipermanent mount.
3. Cut the edge cleanly with a razor blade.

Figure 19. Application of Scotch Magic Tape labels. Photo by David Wagner.

Jan-Peter Frahm (Bryonet 11 December 2013) suggests another alternative for quick, long lasting labels using a computer and Brother P-Touch labelling machine connected through a USB port (Figure 20). This printer also works as a printer for MS Word so that one can make a template in Word. The labels are superior because they are plastified and do not flake off as is often the case with other labels. The print is superior to handwriting for legibility.

Figure 20. Slide label made using Brother P-Touch labelling machine. Photo by Jan-Peter Frahm.

Des Callaghan (Bryonet 11 December 2013) suggests using a laser printer, then cutting labels to size and attach them with PVA glue or double-sided sticky tape (Figure 21). The labels can be sprayed with hair lacquer **before** sticking them to the slide to protect them from abrasion.

Figure 21. Slide labels using a laser printer, PVA glue, and hair lacquer. Photo by Des Callaghan.

Richard Zander (Bryonet 11 December 2013) considers all commercial slide labels to be inadequate, coming off the slide within a few decades. He feels all slides should be kept for students to use in the future. This is especially important for rare collections with little material. Zander cuts rectangular labels from buffered paper and stores a supply in a box. When needed, they are glued on a slide with Elmer's white glue (polyvinyl acetate – "a superb glass adhesive"). This can also be used to re-attach labels that fall off. The glue is kept half cm deep in a balsam applicator bottle so that no glue collects around the edges of the top and is has a glass applicator rod in it. The glue should be slightly diluted with water and the lid of the balsam bottle should be coated with mineral oil on the ground glass to make a better seal. Only a tiny drop of glue should be rubbed on the back of label and label pressed on the slide. Information is added to the label with permanent, non-fading ink, but print carefully so it can be read by people whose first language uses a different alphabet.

Slide Storage

Mounting media of "permanent slides" tend to settle if the slide is placed on its side. Hence, horizontal storage is usually essential. Richard Zander (11 September 1998) makes "trays" using corrugated cardboard. Strips of cardboard are glued across the tops and sides, and one down the middle. This provides the right space to keep slides lined up and the height keeps the next "tray" from making contact with the slide. He recommends gluing a small tag on the bottom edge of the "tray" that tells the contents of that group of slides.

Wagner stores his slides on trays the size of plant press boards or herbarium sheets so they fit on shelves of a standard herbarium cabinet. Three rows can be placed from end to end. Its durability was tested when he dropped a tray of slides in a parking lot. The tray was sandwiched between cardboard press boards so when the bundle hit the pavement upside down, only one slide was thrown out and broken. The rest survived unscathed even though tumbled.

Storage trays need not be made of expensive material. Some bryologists use cardboard trays, although they may suffer loss of rigidity if they get wet. Wagner is currently using foam core board. It helps to line the bottom with blotting paper or similar absorbent material to help absorb any glycerine that travels there by capillarity. It also helps to super glue borders and dividers that are 1.8 cm wide by .4-.5 cm thick as a means of keeping the slides in place.

Preserving Bryophyte Plants for Dioramas

The following advice for preparing dioramas is from Roxanne Hastings and Donna Cherniawsky, Curators of Botany, Royal Alberta Museum, Canada. The recipe came from a display tech, Ludo Bogart, long since retired and where he got it from nobody knows, including him.

Field Collections

1. It is critical that you get the plants into the tank as fresh as possible… especially with vascular plants, less important for bryophytes and lichens but absolutely critical for gymnosperms. Large plastic bags work well (museum specimen bags of heavy poly count – not Safeway Ziploc) or sheets; wrap the plants into them in the field and put them into the largest thermos tubs available, with ice packs. For conifer shrubs and long branches, you can wrap them in plastic and put them on top of blocks of dry ice to get them back home from the field. You have maybe two days at most to get the plants from field and into the vat. Having walk-in refrigeration at both a field station and at the museum will go an awful long way to the success of preservation. Putting the plants into Tupperware-like containers and storing them in refrigeration will buy you several days time. Getting conifers in the tank the same day you get back from the field, no matter how late, is important. The other material, if stored in refrigeration, can wait a day or two.
2. Hastings and Donna Cherniawsky would go into an area and spend the first few days just scouting the sites and tagging all the spots where they wanted to collect. And then in the last 2-3 days they went back to all the sites and collected the stuff in a mad rush.
3. What works superbly for collecting sheets of mosses is large plastic under-the-bed storage trays with snap-on lids. The depth is about perfect for the height of mosses and the trays can be easily stacked on top of one another, which makes transport in the back of a van or minibus that much easier. Also get a number of smaller plastic containers to pick up smaller samples of evergreen shrubs and specific mosses that you can weave into your display when you build your diorama. These will make for a more realistic forest floor, and having the specimens in separate tubs

makes it easier to find them when you are building your display. Tupperware will become your friend for this project.

4. Collect at least twice as much as you think you are going to need for the display. Some specimens will just fall apart in the processing or will just not look as good as you thought they would when you saw them in the field. Having more diversity available will help you create better displays. Any extra material will find a happy home in a teaching collection. People absolutely love handling specimens that they can see on display in your gallery and they make for gorgeous open house/public appreciation days material.

Preservation Protocol (From Roxanne Hastings, pers. comm. 11 October 2012)

1. Assemble the equipment:
   stainless steel tank with a lid
   perforated stainless steel paddle or spoon
   stainless steel wire mesh
   roll of stainless steel wire
   fume hood
   source of hot tap water
   sink hose
   big funnel
2. Assemble personal safety equipment:
   Wrap around eye goggles
   Nose and mouth mask – medical or industrial
   Long rubber gloves
   Rubber boots – safety toes if working with barrels of liquids
   Knee length lab coats
   Breathing mask is good idea if working with industrial volumes

   NB you must wear proper safety equipment. Acetone and alcohol are dangerous. At the minimum you need safety goggles for your eyes and you should have an eye rinse station in the area and wear elbow length rubber gloves. With big tanks you need rubber boots, maybe even safety toes depending if you have to roll drums yourself. Breathing masks are advised for big jobs. You must work in a ventilated room and the tank must be used under a fume hood. The process sounds a lot worse than what it is. It becomes routine. But you must pay attention to safety. Cover your eyes and cover your mouth and nose. There will be splashing and it will hit you in the face. A trip to an industrial safety supply shop will easily resolve your concerns.

3. Prepare the preservative:
   1 part Acetone
   1 part Isopropyl Alcohol at 70%
   2 part Glycerine

   The acetone evaporates the water in the plants, whereas the alcohol and glycerine enter. The alcohol preserves the plants and the glycerine gives them their lifelike feel and luster. Acetone can remove chlorophyll color and cause some species to become black or grey. Fortunately, mosses and lichens usually came out unscathed and required no touch up. To ensure accurate color rendition, take photographs in the field with color charts and also write down the color numbers so you can match the colors later. You can use standard paint chips available free at paint shops to match colors.

4. These fluids are all **thoroughly** mixed together at once. This takes some effort because they have significant density differences. For large batches, you can use perforated steel shelves built so that they can be put into a large tank on a ledge built all around the inside face of the tank at half height. This allows two layers of specimens without them all piling up in the bottom of the tank.
5. Moss specimens will easily fall apart in a big tank so it helps to wrap the sheets of mosses in stainless steel mesh - what concrete people use to provide structure for a sidewalk when they pour concrete into it. You will need a strong set of metal clippers to cut the mesh and a long roll of wire to stitch the mesh together once you have wrapped your specimens. The mesh is reusable. Wear leather work gloves because the wire mesh is really sharp once it is cut.
6. If you are doing a big job and need to order your chemicals by the drum, then you will need a stainless steel hand pump to get the fluid from the barrel into the tank. You must ground the pump with a wire to prevent friction sparking when you pump. Plastic pumps won't spark but they are useless for pumping viscous liquids like glycerine and will melt in Acetone. And obviously you cannot use electric pumps. If you are doing a smaller job you can buy the supplies by the liter and just dump it into the tank. Then you won't have sparking issues.
7. It takes a lot of stirring; a perforated stainless steel spoon or paddle works best. Plastic will eventually melt in the acetone and is not strong enough to stir glycerine. The perforations provide better mixing and glides more easily through the glycerine.
8. Put your specimens into the tank and keep pushing them down. They will float for a few hours to a few days while they absorb the fluid and the air gets beat out of them. Gently stir them until they settle. In a big tank you may have to come back the next day and gently stir them again. Let them sit for a few weeks. Mosses go through the process in a matter of weeks. Small trees or branches in the order of a meter or so long will take a few months.
9. Put a lid on the tank; it should also be stainless steel. You MUST do this in a fume hood. Smaller tanks can be put in a lab fume hood.
10. Then you have to fish them out. A 3 m long stainless steel rod with a hook on it to grab the mesh works well. Large plants will be really, really heavy! Lift them out and let them drain into the tank. Then you have to rinse them, still in the screens, with very hot tap water. Rinse them until the water runs clear and not brown. Don't let this water go down a drain! Rinse them back into your now empty chemical drums. You can use a big plastic funnel with screens in it; put the funnel over the drums and rinse into that.
11. Once the plants are drained, open up the screens and lay out the specimens **in the fume hood** to dry. This will take at least a week. Keep checking.

12. Now your plants are ready to process. They will be lustrous and flexible with various amounts of fading that may require some airbrushing – or not. Some specimens still look very good after 40 years. Specimens that were processed in 2008 were still in perfect condition in 2012 and you could still smell the aroma of the sap of the pine trees. All the pines still have their needles and are flexible (2012). You can use leftovers for hands-on teaching with school kids and the public; they are still perfectly life like, in fact they are tougher because they are a tiny bit like rubber cement.

Preserving Liverworts

Despite Rob Gradstein's claim that thallose liverworts can be dried like mosses (see chapter on Field Taxonomy and Collecting Methods in this volume), Wagner considers preservation in liquid a necessity for examination of morphological characters. He recommends using rubber-topped vials used for drawing blood and reports that some are 20 years old, but have not lost any appreciable amount of liquid.

Susan Moyle Studlar (Bryonet 20 September 1999) has observed that some of the thallose liverworts retain their green color and are easy to work with after air drying in a plant press. She has had success with this treatment of species of ***Conocephalum*** (Figure 24), ***Pellia*** (Figure 23), and ***Pallavicinia*** (Figure 22).

Figure 22. ***Pallavicinia lyellii***, a liverwort that can be preserved by drying. Photo by Jan-Peter Frahm.

Jesús Muñoz (Bryonet 19 February 1999) suggests two formulas for preserving liverworts:

10:1:1:8 96% ethanol : glutaraldehyde : glycerol : water

or

1:1:18 glutaraldehyde : glacial propionic acid : 70% ethanol

Muñoz reports that you can use formaldehyde instead of glutaraldehyde, but that glutaraldehyde gives better results. The bottles must close tightly or the ethanol evaporates. Unfortunately, the mixtures degrade chlorophyll and lipids, so although morphology and anatomy remain as in live plants, oil-bodies and natural color disappear. As suggested in the chapter on Field Taxonomy and Collecting Methods in this volume, it is a good idea also to keep a herbarium sample cross-referenced to the liquid sample so that there is still material for DNA or chemical analysis.

Figure 23. ***Pellia endiviviifolia*** near Swallow Falls, Wales. This liverwort can be preserved by drying. Photo by Janice Glime.

Figure 24. ***Conocephalum conicum***, a liverwort that can be preserved by drying. Photo by Dick Haaksma.

David Wagner (Bryonet 19 September 1999) agrees that these two formulas work well. He advises against Pohlstoffe for thallose liverworts and compares working with the revived specimens to working with boiled lettuce. Even leafy liverworts do not fare well in Pohlstoffe because the oil bodies disintegrate in that and other wetting agents. The oil bodies sometimes survive drying if water, but no wetting agent, is added to rehydrate them. Wagner uses FAA (formalin-acetic acid-alcohol), which is similar to the suggestions of Muñoz except for the presence of 5% acetic acid and lack of glycerine. But Wagner points out that the

glycerine is important to keep the tissues soft if they accidentally dry out. The downside of glycerine is that it can interfere with the embedding process if one wants to prepare the specimen for microtome sectioning.

David Wagner (Bryonet 5 November 2022) uses a 50:50 mix of denatured alcohol and distilled white vinegar as a liquid preservative. The denatured alcohol is available at hardware stores in the paint section where paint thinners, paint strippers, solvents, *etc*. are found. A common one has the brand name Klean Strip and actually has a high percentage of methanol, which is why it is a good glass cleaner and marine stove fuel. Of course, it is really poisonous if consumed. The vinegar is 5% acetic acid, from a grocery store; it helps preserve proteinaceous cell structure. The advantage of this method is that it is cheap and readily available to most people. Wagner used this method to preserve ***Riella americana*** with sporophytes (Figure 25).

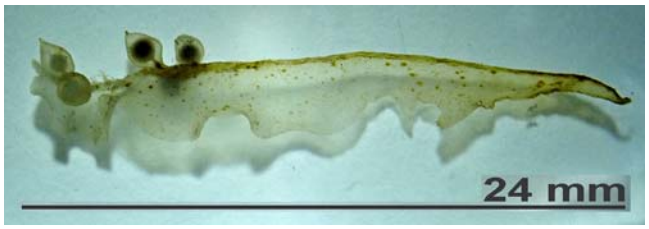


Figure 25. ***Riella americana*** with sporophytes, preserved with methanol and vinegar. Photo by David Wagner, with permission.

Michael Christianson (Bryonet 19 September 1999) raised the possibility of using **lyophilization** (freeze-drying), suggesting it should keep such important organelles as chlorophyll and oil bodies intact. If the material is put into ampoules after lyophilization, these could be sealed. If the ampoule is further treated by replacing the air with nitrogen, Christianson predicts that DNA would be preserved for decades.

One of the problems in preservation is that such specimens require maintenance about once a year to replace the liquid that has evaporated from them. Angela Newton (Bryonet 20 September 1999) suggested that this labor can be greatly reduced by placing a group of vials in a larger jar that can easily be topped off. Furthermore, it will provide a head of liquid that will last much longer than that of a small vial. This also helps to reduce swirling motion that can damage small specimens during the refill process.

Kronestedt and Echlin (1982) suggested freeze-drying the thallose liverwort ***Ricciocarpos natans*** (Figure 26) instead of acetone or ethanol dehydration in preparation for critical point drying for scanning electron microscopy because freeze-drying causes less cell collapse.

Figure 26. ***Ricciocarpos natans*** thallus, one whose cells collapse in acetone or ethanol. Photo by Ralf Wagner <www.dr-ralf-wagner.de>.

Summary

Permanent slides usually require use of a mounting medium. Ideally, these must dry quickly, be effective in clearing, be durable, and be easily available. Older mounts used glycerine, other mountants include gum arabic, Hoyer's solution, gum chloral, DMHF (5,5-dimethyl Hydantoin Formaldehyde), PVA, Polyvinyl Lactophenol, Aquamount, Kleermount, Fluoromount-G, Gray-Wess Mountant. Slides can also be sealed with a lutant like fingernail polish, including those using the double-coverslip method. Most of these methods permit repair when the slide dries out too much. Storage usually must be flat to prevent movement of the mountant and specimen.

Caution is needed in selecting a mountant. Chloral hydrate (in Hoyer's solution) is toxic, and some mountants distort the cells or cause plasmolysis.

A mix of glycerol and lactic acid can be used to clear specimens. Liverworts may require special preservation, but some thallose specimen can simply be dried.

Acknowledgments

I appreciate all those who have given me permission to use images and those who have put them on the web in the public domain.

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