

CHAPTER 2-2a

LABORATORY TECHNIQUES: SLIDE PREPARATION AND STAINS

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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.

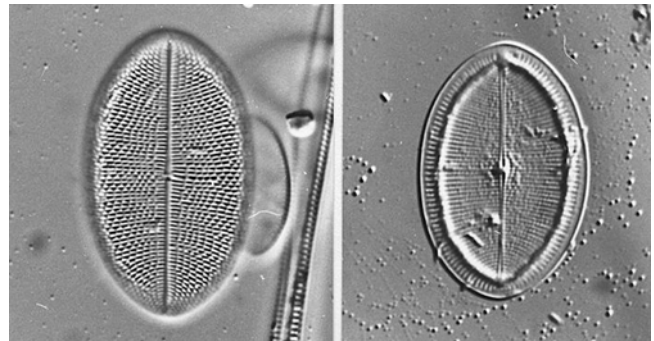


Figure 4. Both valves of *Cocconeis placentula*, a common diatom that imbeds itself into the cell wall of aquatic bryophytes. Photo by Pauli Snoeijs, Nordic Microalgae <www.nordicmicroalgae.org>, through Creative Commons.

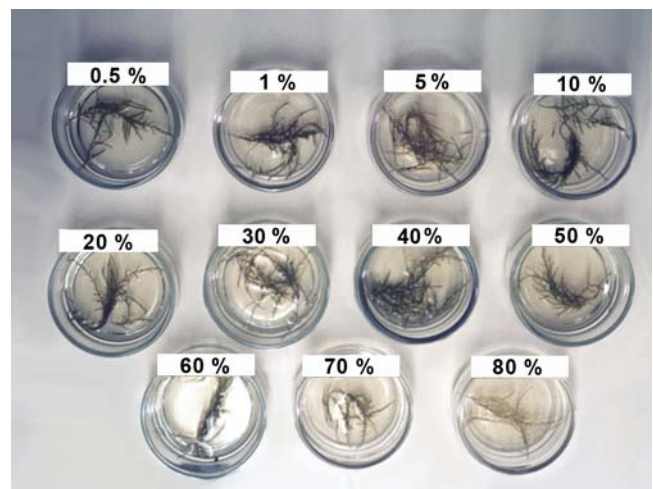


Figure 5. Experiment on cleaning *Fontinalis* sp. with household hydrogen peroxide at various concentrations. Note the bleaching at 70 and 80%. Photo by Janice Glime.

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 Thekathyl, Bryonet 12 May 2010)

Another solution to wetting bryophytes is to use **soap** or detergent as a wetting agent. Tom Thekathyl (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 Thekathyl, Bryonet 12 May 2010)

Tom Thekathyl 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.

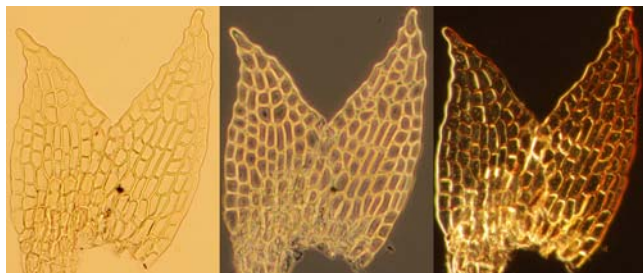


Figure 10. Cleared leaves of *Anthelia juratzkana*. **left:** brightfield, **mid:** phase contrast, **right:** dark field. Photos by Des Callaghan, with permission.

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 Permunt 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.

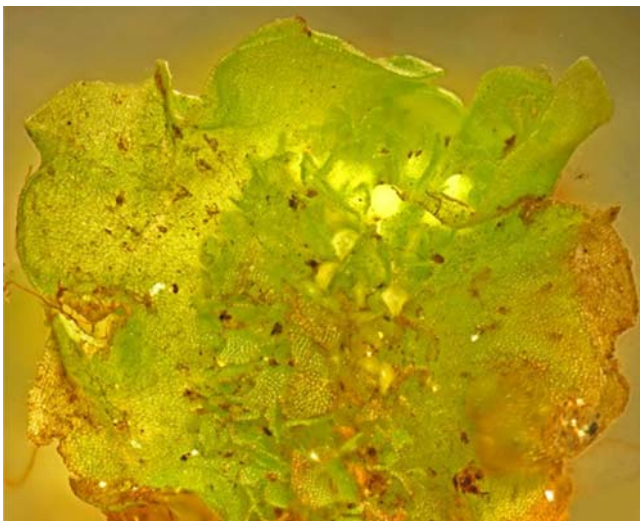


Figure 11. *Moerckia blyttii* fresh plant. Photo by David Wagner.



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

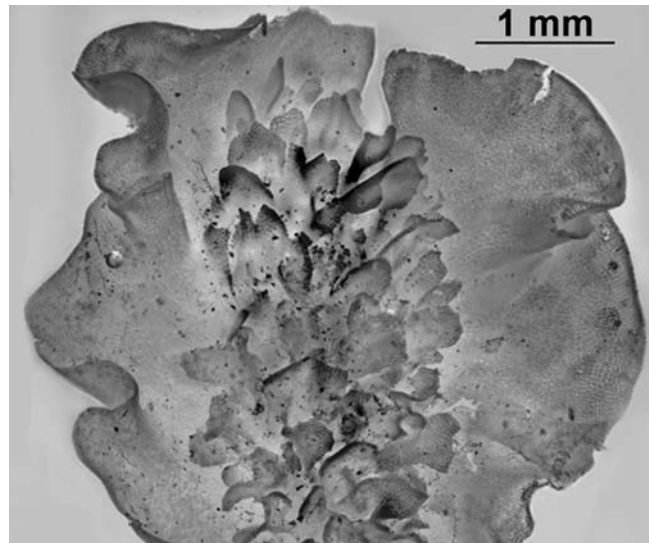


Figure 13. *Moerckia blyttii* cleared and stained, grey scale. Photo by David Wagner.

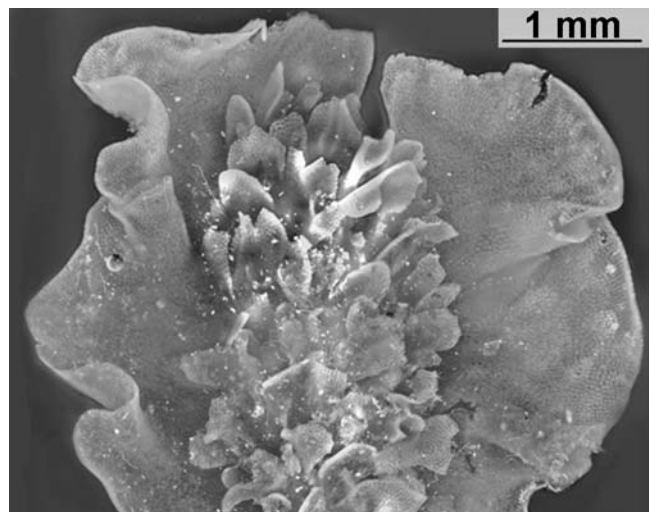


Figure 14. *Moerckia blyttii* cleared and stained; gray scale positive image converted to negative. Photo by David Wagner.

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 Thekathyl (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).

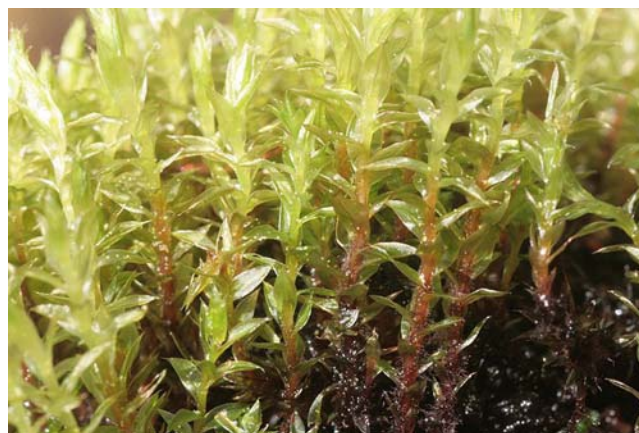


Figure 16. *Bryum pseudotriquetrum*, a species Kawai cut in 15 μm sections. Photo by Hermann Schachner, through Creative Commons.

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 μ m** 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.

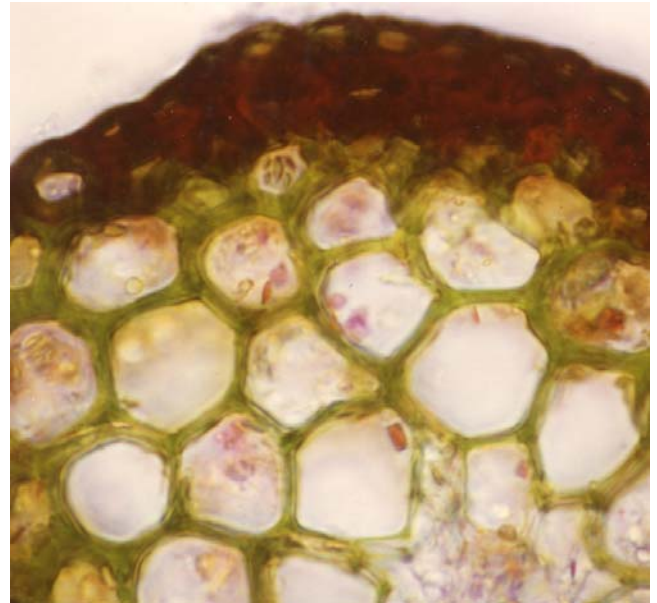


Figure 25. *Bryoxiphium* sp. stem cross section stained with **eosin** for 2 hours and **methyl green** for 30 seconds. Photo courtesy of Isawo Kawai.

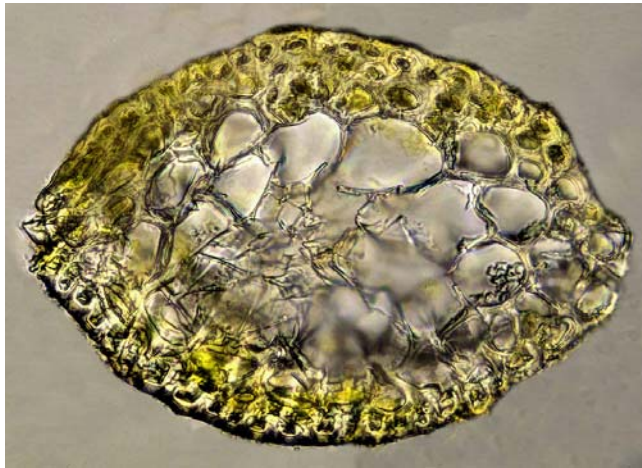


Figure 23. *Fissidens bryoides* stem cs, unstained. Photo by Dick Haaksma, with permission.

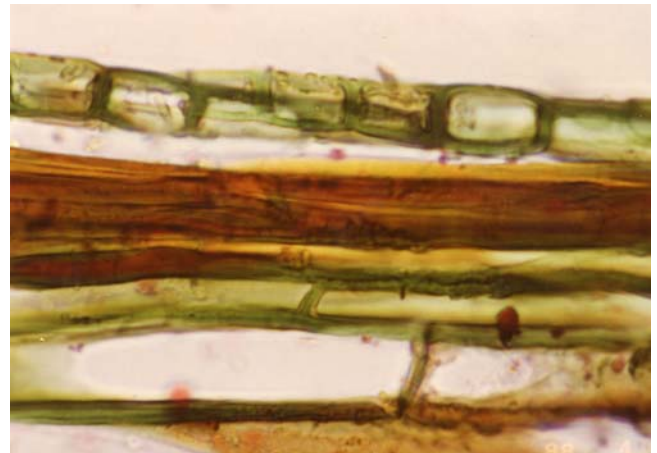


Figure 26. *Bryoxiphium* sp. stem longitudinal section stained with **eosin** for 2 hours and **methyl green** for 30 seconds. Photo courtesy of Isawo Kawai.

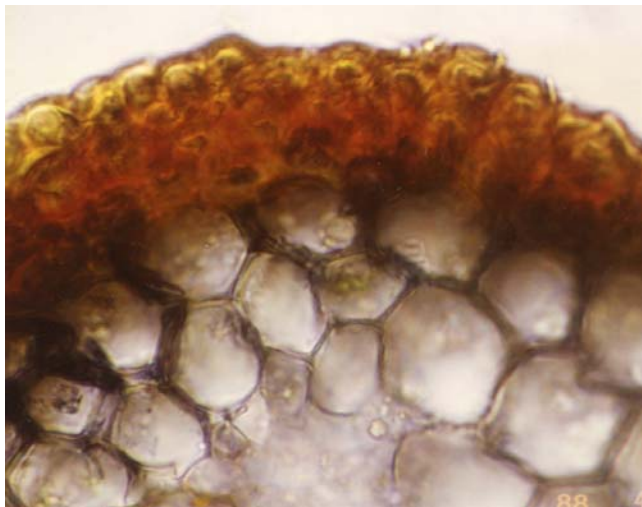


Figure 24. *Bryoxiphium* sp. stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

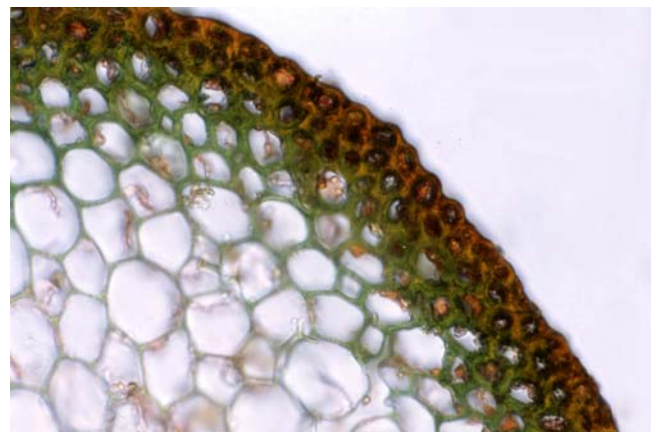


Figure 27. *Fontinalis antipyretica* stem cross section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with 0.2 g per 100 cc **eosin** for 50 minutes. The blue-green/green color clearly shows the inner layer of "epidermal" portion of the stem. Photo courtesy of Isawo Kawai.

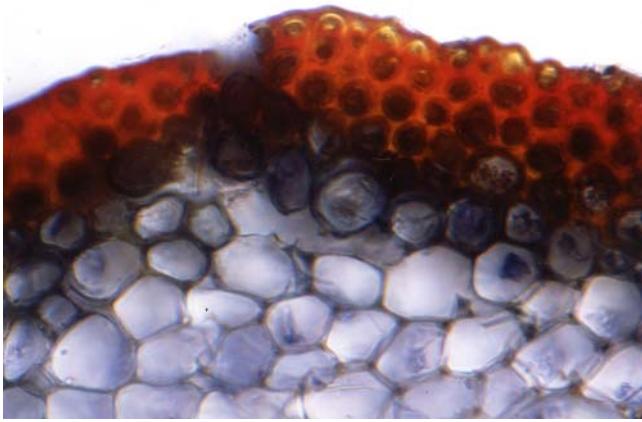


Figure 28. *Fontinalis antipyretica* stem cross section stained in **aniline blue** for 20 minutes. Photo courtesy of Isawo Kawai.

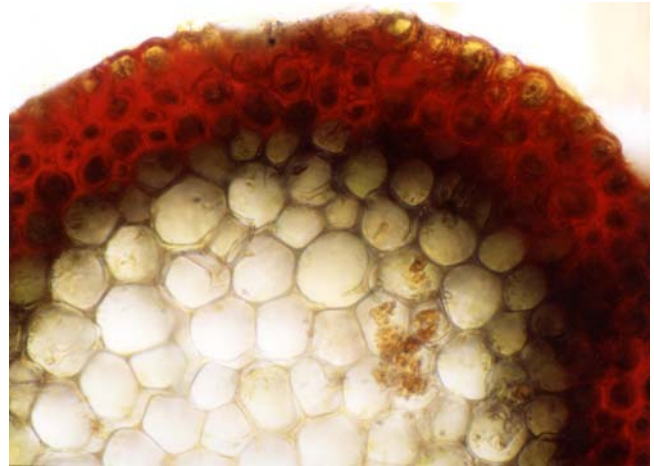


Figure 31. *Fontinalis antipyretica* stem cross section stained in **aniline blue + eosin** for 1 hour. Compare this to the previous picture (Figure 32) to see differences that can occur under the same staining protocol. These differences may relate to age of the tissues or possibly the habitat. Photo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

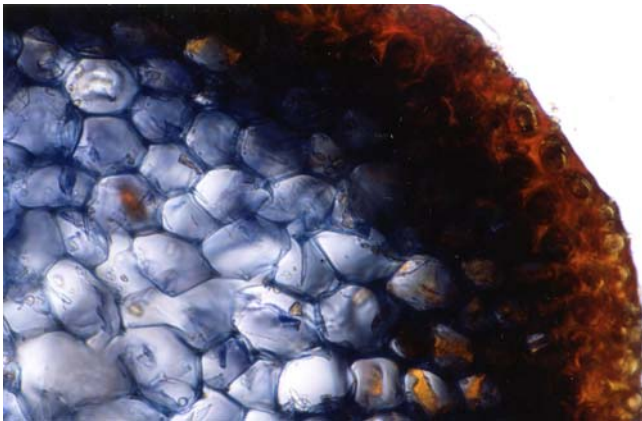


Figure 29. *Fontinalis antipyretica* stem cross section stained with **aniline blue** for 30 minutes. Photo courtesy of Isawo Kawai.

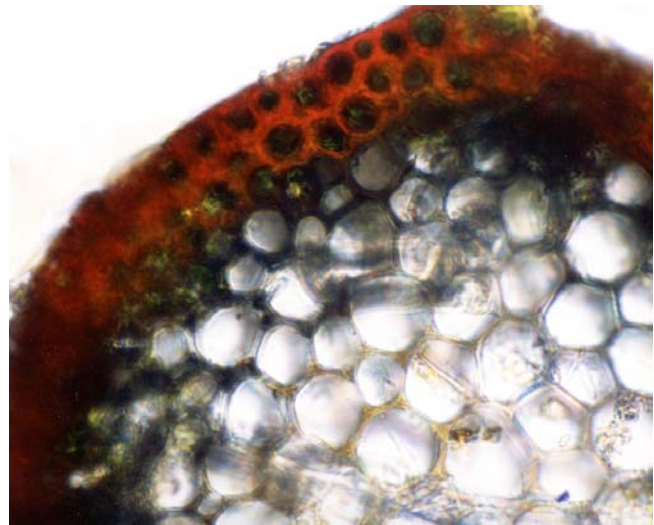


Figure 32. *Fontinalis antipyretica* stem cross section stained in **aniline blue + eosin** for 1 hour. Photo courtesy of Isawo Kawai.

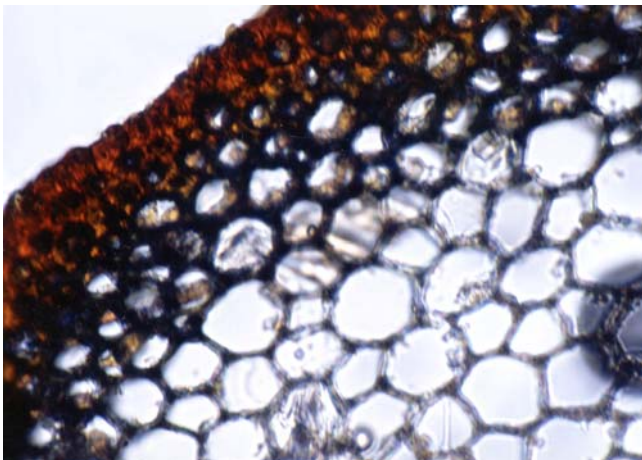


Figure 30. *Fontinalis antipyretica* stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

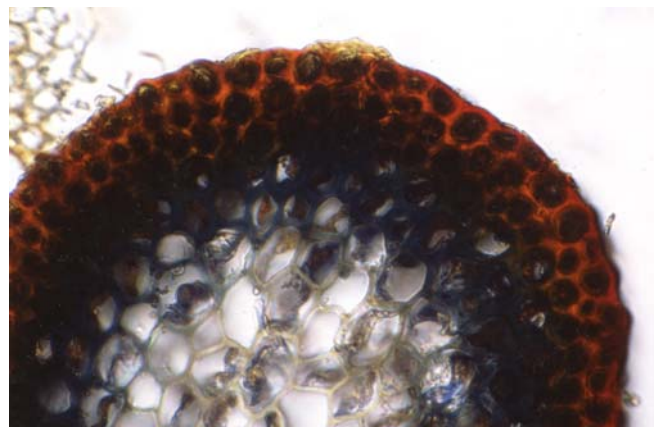


Figure 33. *Fontinalis gracilis* stem cross section stained with **aniline blue** for 5 minutes. Photo courtesy of Isawo Kawai.

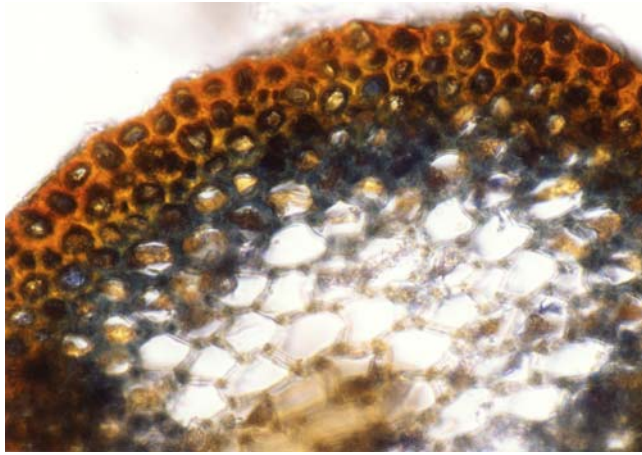


Figure 34. *Fontinalis gracilis* stem cross section stained with **aniline blue** for 5 minutes. Photo courtesy of Isawo Kawai.

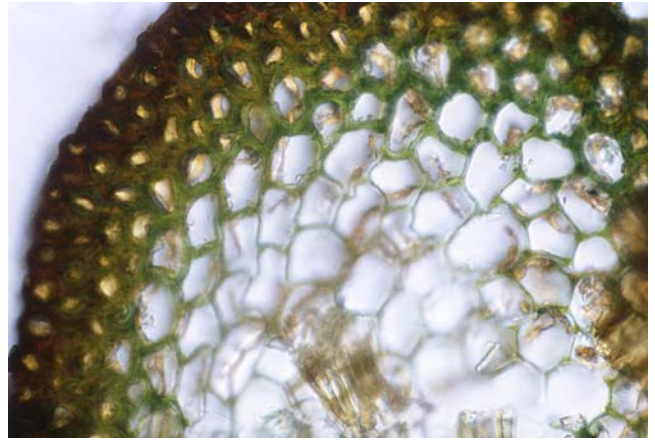


Figure 37. *Fontinalis gracilis* stem cross section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with **methyl green** + 0.2 g per 100 cc **eosin** for 1 hour. Photo courtesy of Isawo Kawai.

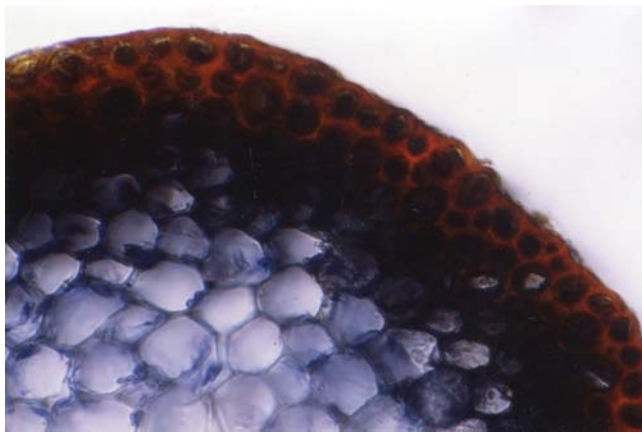


Figure 35. *Fontinalis gracilis* stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

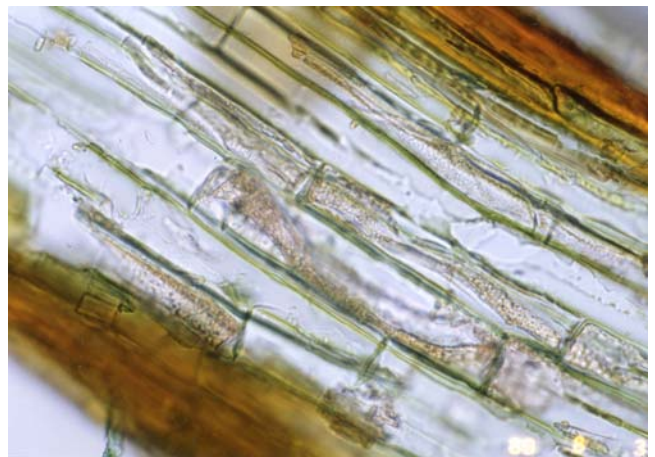


Figure 38. *Fontinalis gracilis* stem longitudinal section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with **methyl green** + 0.2 g per 100 cc **eosin** for 15 minutes. Photo courtesy of Isawo Kawai.

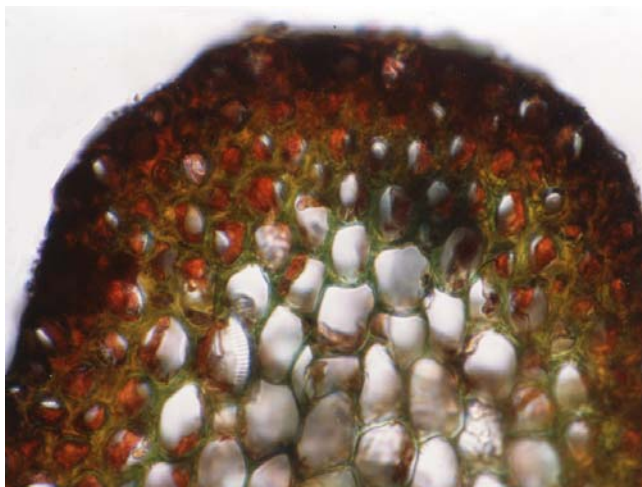


Figure 36. *Fontinalis gracilis* stem cross section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with **methyl green** + 0.2 g per 100 cc **eosin** for 15 minutes. Photo courtesy of Isawo Kawai.

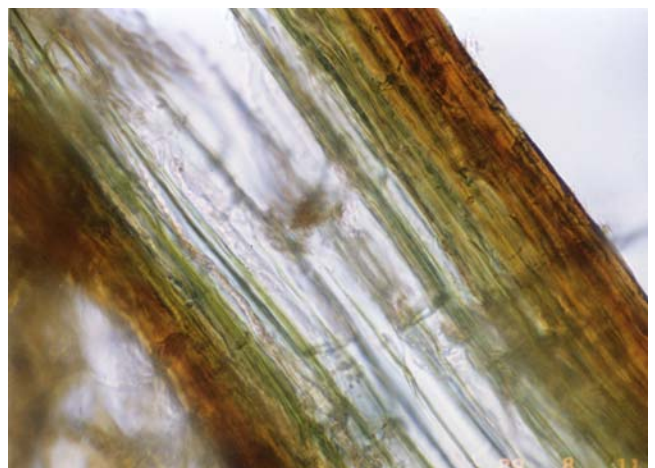


Figure 39. *Fontinalis gracilis* stem longitudinal section stained with 0.005 g per 100 cc **methyl green** for 10 seconds, then stained with **methyl green** + 0.2 g per 100 cc **eosin** for 1 hour. Photo courtesy of Isawo Kawai.



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.

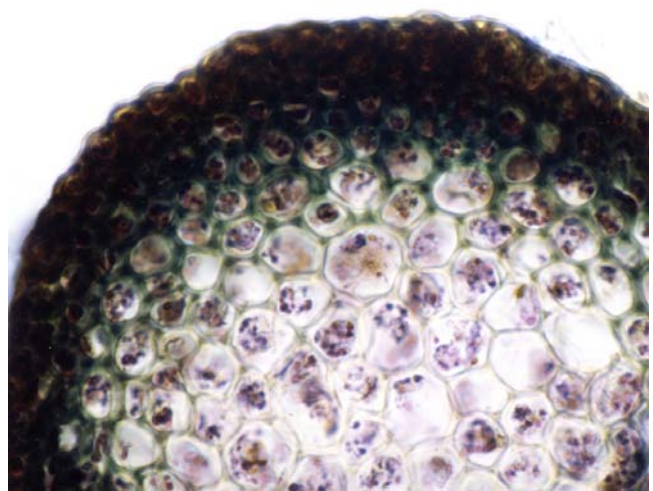


Figure 43. *Fontinalis hypnoides* stem cross section stained with **aniline blue** + **eosin** for 7 hours. Photo courtesy of Isawo Kawai.

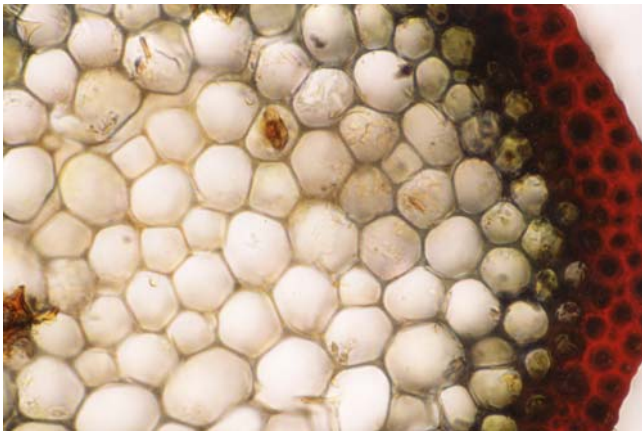


Figure 41. *Fontinalis hypnoides* stem cross section stained with **aniline blue** for 30 minutes. Photo courtesy of Isawo Kawai.

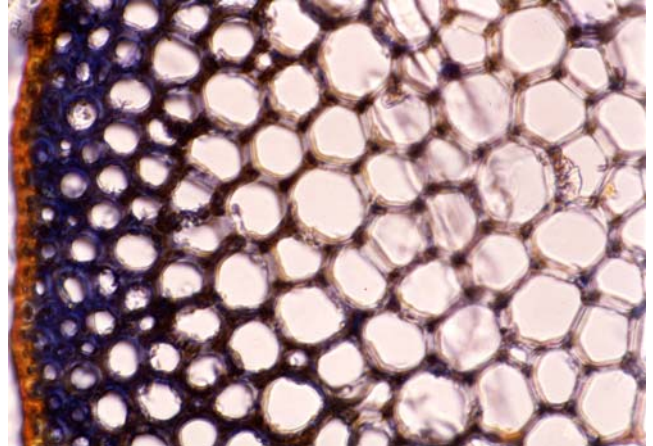


Figure 44. *Hylocomium* sp. stem cross section stained with **aniline blue** for 3 hours. Photo courtesy of Isawo Kawai.

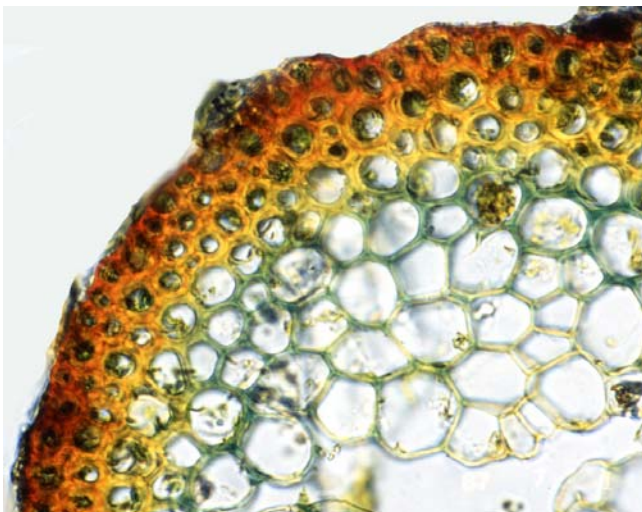


Figure 42. *Fontinalis hypnoides* stem cross section stained with **aniline blue** + **eosin** for 3 hours. Photo courtesy of Isawo Kawai.

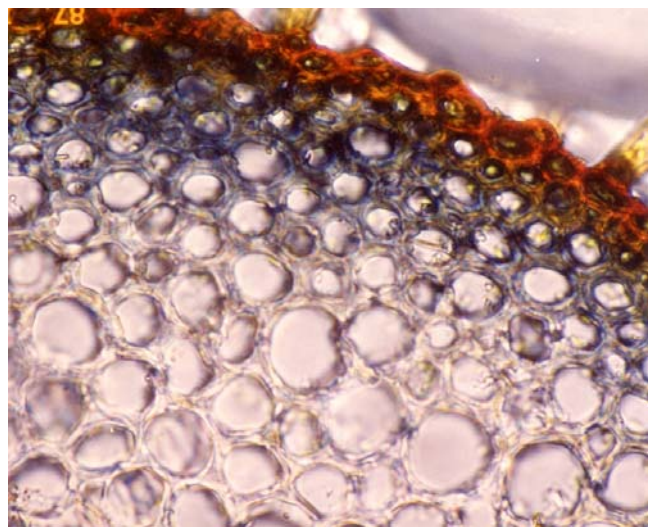


Figure 45. *Hylocomium* sp. stem cross section (5 μ m thick) stained with **aniline blue** + **eosin** for 2 hours. Photo courtesy of Isawo Kawai.

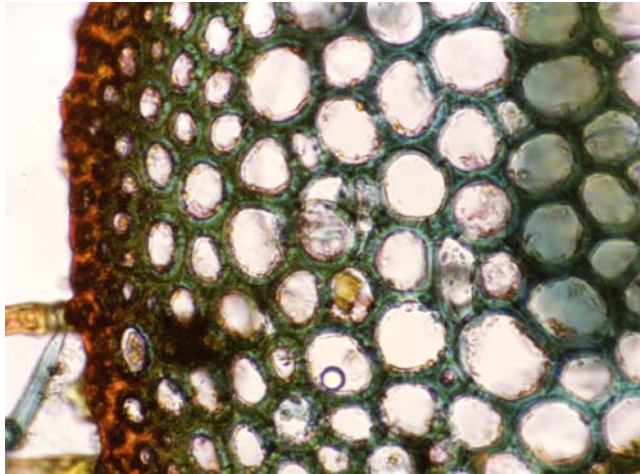


Figure 46. *Hylocomium* sp. stem cross section stained with **eosin** for 1 hour, then with **methyl green** 1 minute. Photo courtesy of Isawo Kawai.

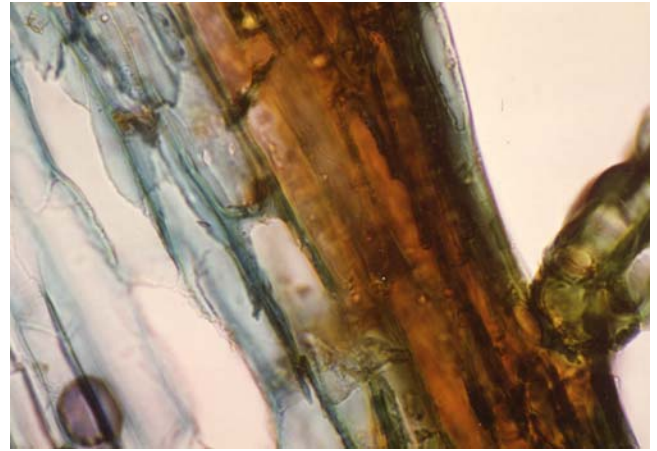


Figure 49. *Hypnum* sp. stem longitudinal section stained with **eosin** for 1 hour, then stained with **methyl green** for 30 seconds. Photo courtesy of Isawo Kawai.

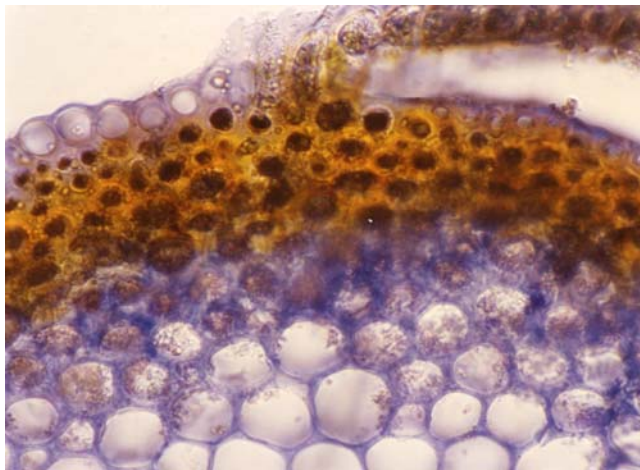


Figure 47. *Hypnum* sp. stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

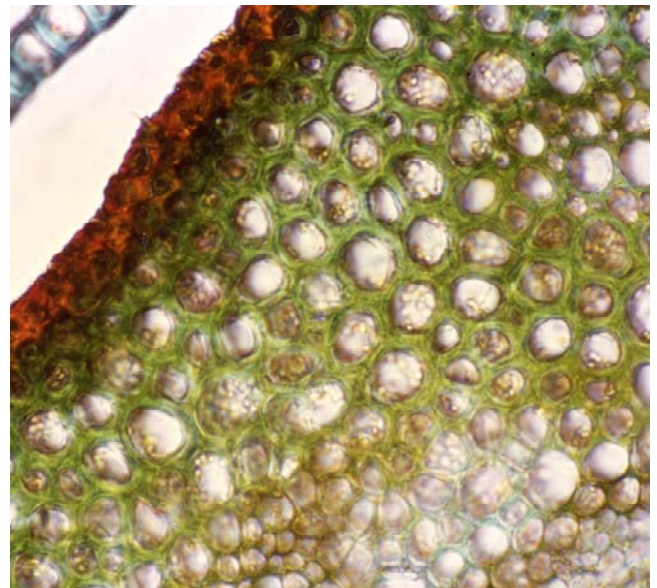


Figure 50. *Polytrichum* sp. stem cross section stained with **eosin** for 1 hour, then stained with **methyl green** for 2 minutes. Photo courtesy of Isawo Kawai.

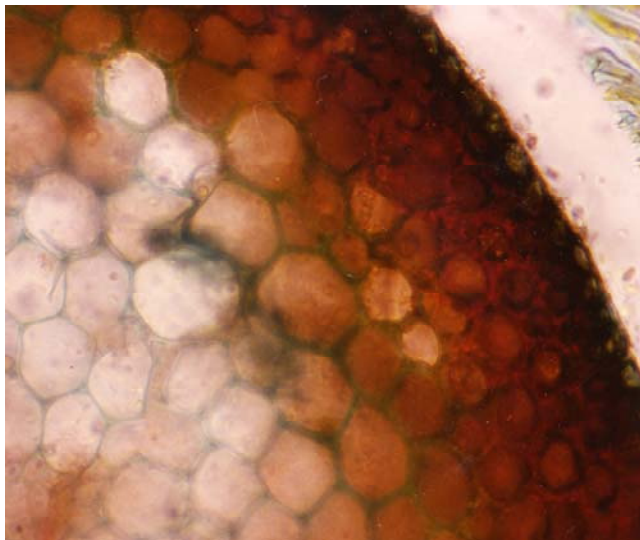


Figure 48. *Hypnum* sp. stem cross section stained with **eosin** for 1 hour, **washed**, then stained with **methyl green** for 0.5-1 minutes. Photo courtesy of Isawo Kawai.

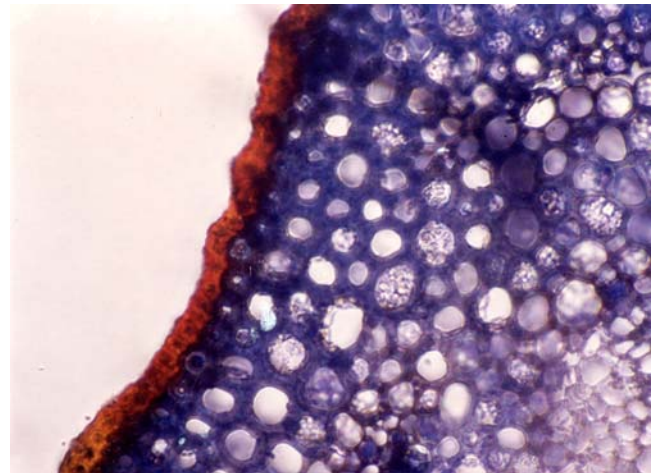


Figure 51. *Polytrichum* sp. stem cross section stained with **aniline blue** for 2 hours. Note the cell inclusions in these cortex cells. Photo courtesy of Isawo Kawai.

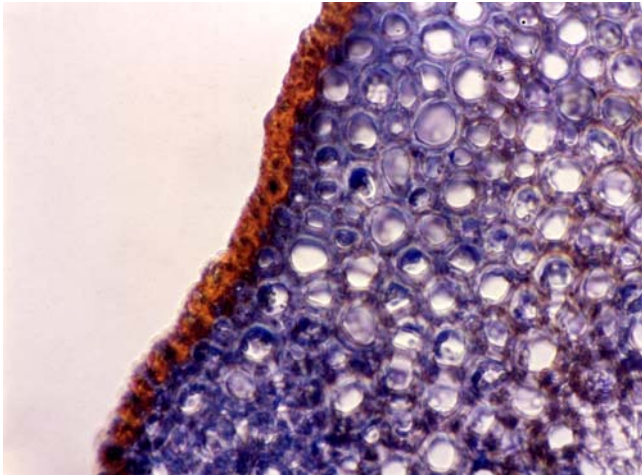


Figure 52. *Polytrichum* sp. stem cross section stained with **aniline blue** for 2 hours. Photo courtesy of Isawo Kawai.

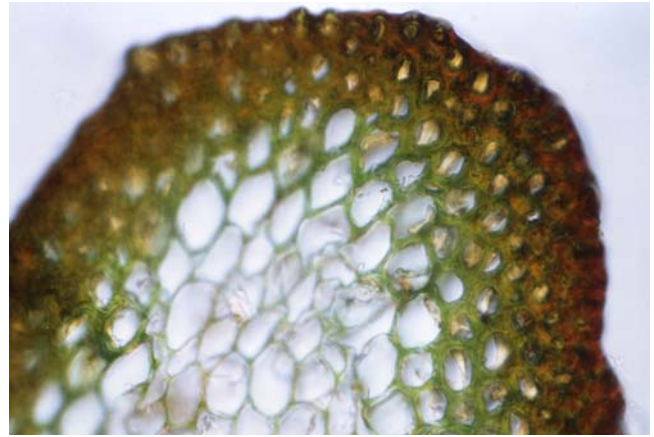


Figure 55. *Polytrichum* sp. stem cross section stained with 0.01g per 100 cc **methyl green** for 50 seconds, then stained with 0.3 g per 100 cc **eosin** for 2 hours. Photo courtesy of Isawo Kawai.

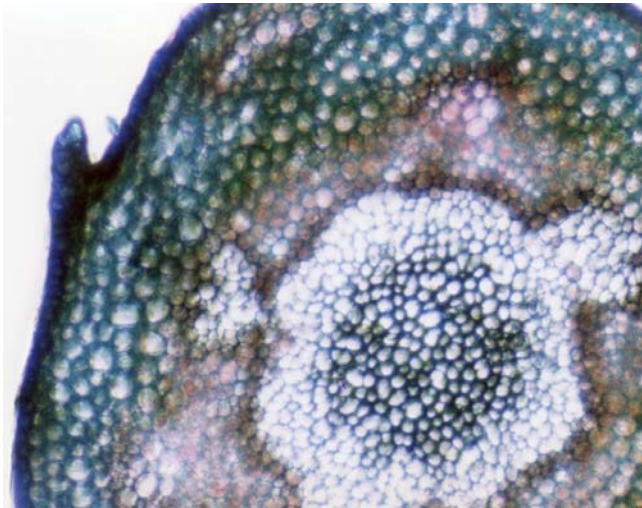


Figure 53. *Polytrichum* sp. 10 µm stem cross section stained with 0.01g per 100 cc **methyl green** for 50 seconds, then 0.3 g per 100 cc **eosin** was added for 2 hours, then **washed** with water. Photo courtesy of Isawo Kawai.

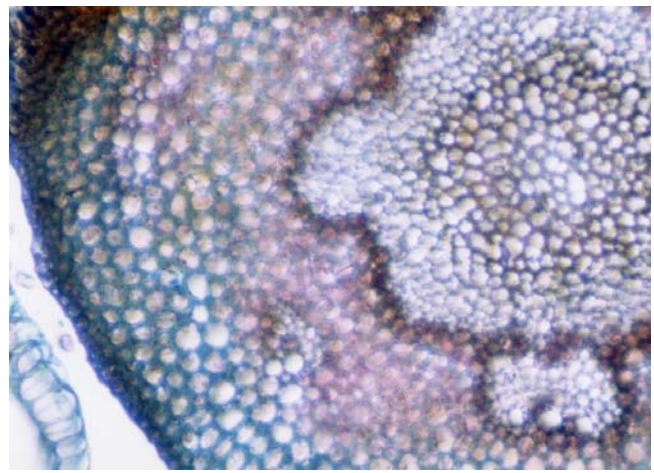


Figure 56. *Polytrichum* sp. stem cross section stained with 0.01g per 100 cc **methyl green** for 3 minutes, then stained with **eosin** for 2 hours. Photo courtesy of Isawo Kawai.

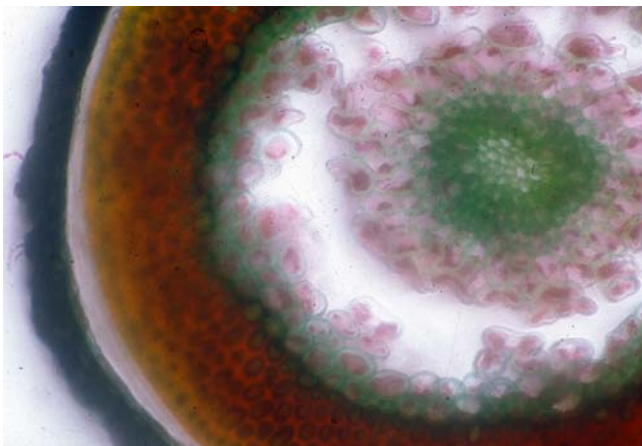


Figure 54. *Polytrichum* sp. stem cross section. The cortex cell walls are blue-green from **methyl green**. The hydrome cells are violet-brown. Photo courtesy of Isawo Kawai.

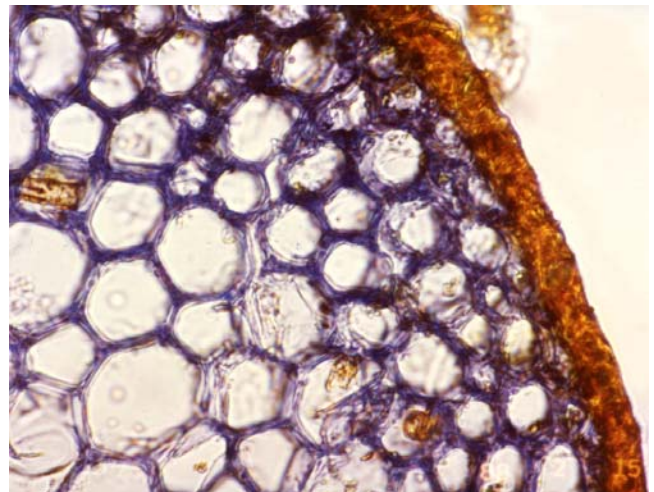


Figure 57. *Rhizogonium* sp. stem cross section stained with **aniline blue** for 1 hour. Photo courtesy of Isawo Kawai.

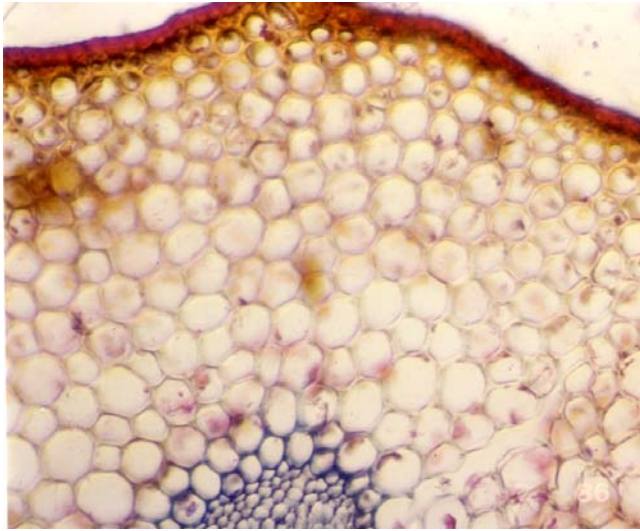


Figure 58. *Rhizogonium* sp. stem cross section stained with **aniline blue + eosin** for 3 days. Photo courtesy of Isawo Kawai.

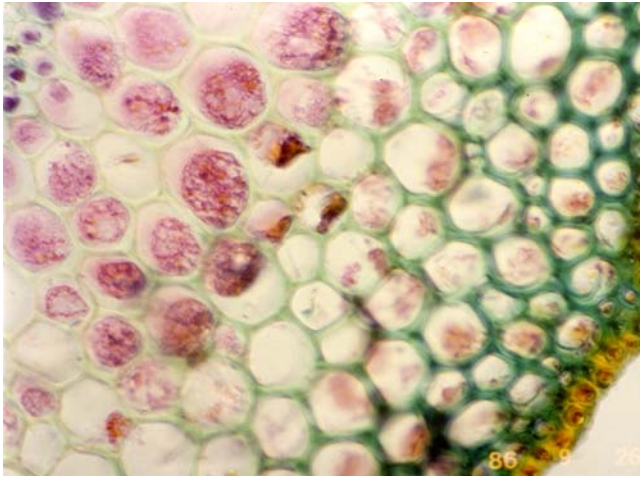


Figure 59. *Rhizogonium* sp. stem cross section stained with **aniline blue + eosin** for 3 days, washed, stained with **eosin** 3 more days, then stained with **methyl green**. Photo courtesy of Isawo Kawai.

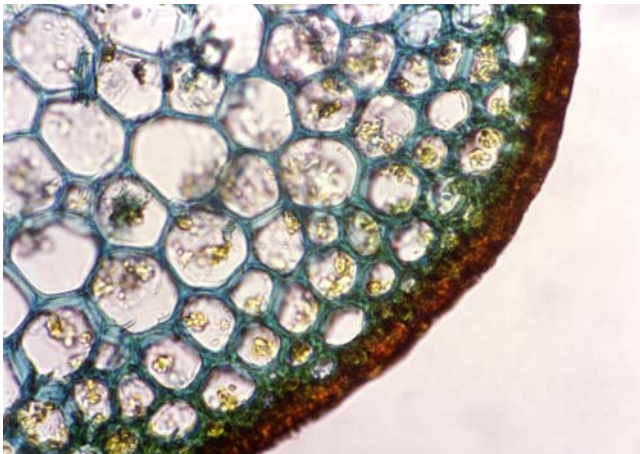


Figure 60. *Rhizogonium* sp. stem cross section stained with **eosin** for 2 hours, washed, then stained with **methyl green** for 1 minute. Photo courtesy of Isawo Kawai.

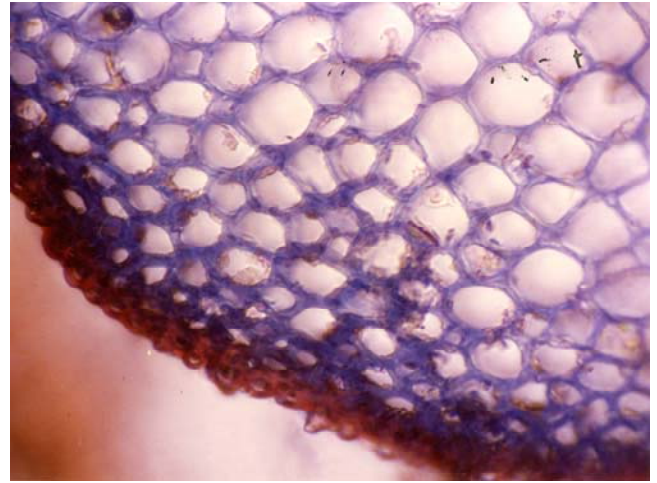


Figure 61. *Thuidium* sp. stem cross section stained with **aniline blue + eosin** for 2 hours. Photo courtesy of Isawo Kawai.

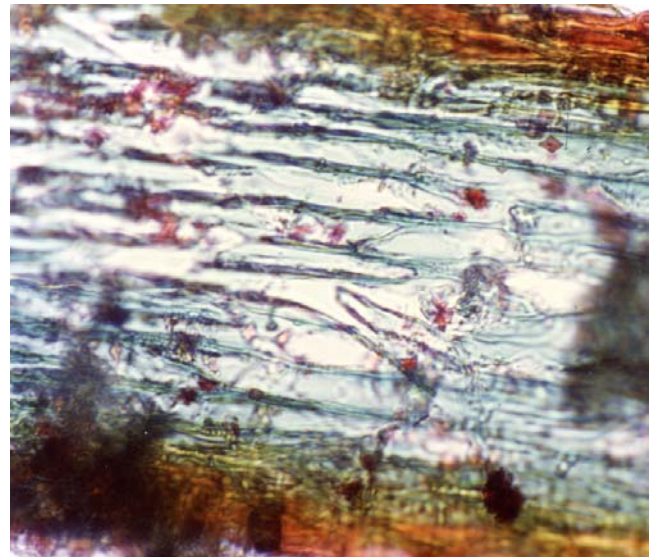


Figure 62. *Thuidium* sp. stem longitudinal section stained with **aniline blue + eosin** for 2 hours. Photo courtesy of Isawo Kawai.

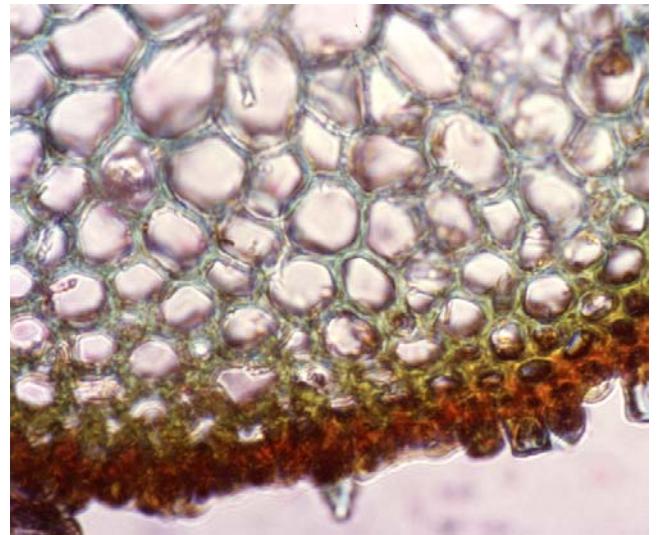


Figure 63. *Thuidium* sp. stem cross section stained with **eosin** for 2 hours, washed, then stained with **methyl green** for 1 minute. Photo courtesy of Isawo Kawai.

Acid Fuchsin

Acid fuchsin has been used to stain a variety of plant, animal, and fungal tissues. Kawai (1980b) used **acid fuchsin**, along with **I₂KI** and **gentian violet** to distinguish the internal anatomy of stems in the **Leucobryaceae** (Figure 64). Using 5 μ m 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 I₂KI 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 μ m stem sections work well. Photo by Martin Hutten, with permission.



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



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



Figure 68. *Hypnum plumaeforme*, a species in which 5 μ m 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 tumorigenic 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 pH 1.0, the dye is green, but in an alkaline solution it is colorless.

Kawai (1980b) used **gentian violet**, along with **acid fuchsin** and **I₂KI** 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

I₂KI – 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** (I₂KI), 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 **I₂KI** 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.

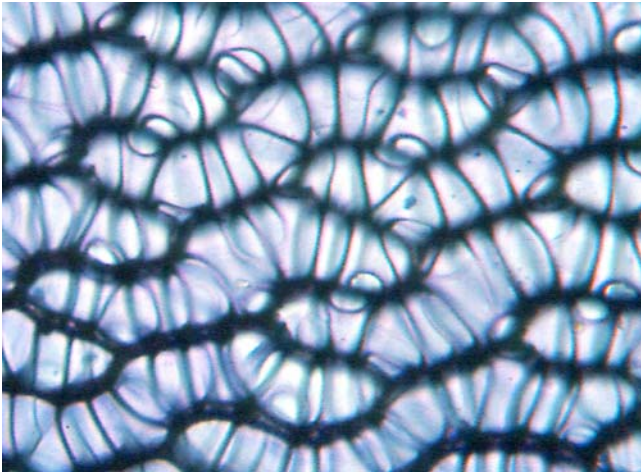


Figure 69. *Sphagnum* leaf cells stained with methylene blue to make pores visible. Photo by Janice Glime.



Figure 71. *Dicranoloma billardieri*. Some members of its genus can be stained with I₂KI. Photo by Michael Lüth, with permission.



Figure 70. *Canaloptygium filiculaeforme*. Some members of its family (*Hypopterygiaceae*) can be stained with I₂KI. 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.

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



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



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



Figure 76. *Sagenotortula quitoensis*, in a genus that stains 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.

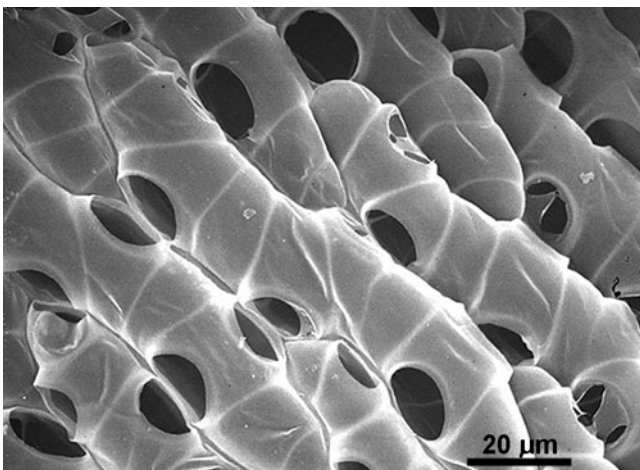


Figure 80. SEM of *Sphagnum* hyaline cells and pores. Photo from Botany Website, UBC, with permission.

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 pyocyanin(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.

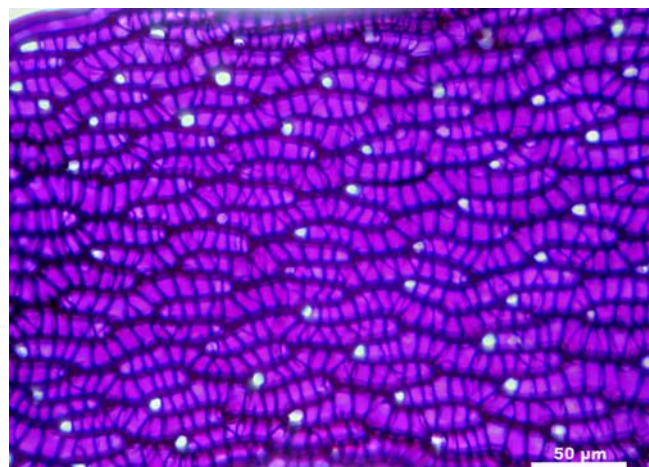


Figure 81. *Sphagnum obtusum* stained for pores. Photo by Ralf Wagner.

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.

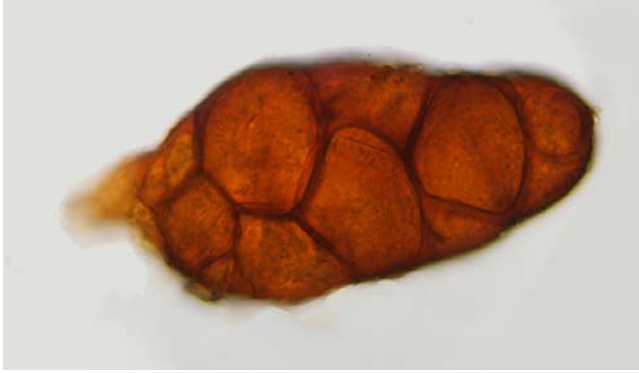


Figure 84. *Pohlia wilsonii* rhizoidal tubers. Photo by Guillermo M. Suárez, with permission.

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 barbuloidea* (Figure 86). Animal tissues exhibit only secondary fluorescence, making it possible to distinguish between animal galls and bryophyte propagules (Nordhorn-Richter 1988).

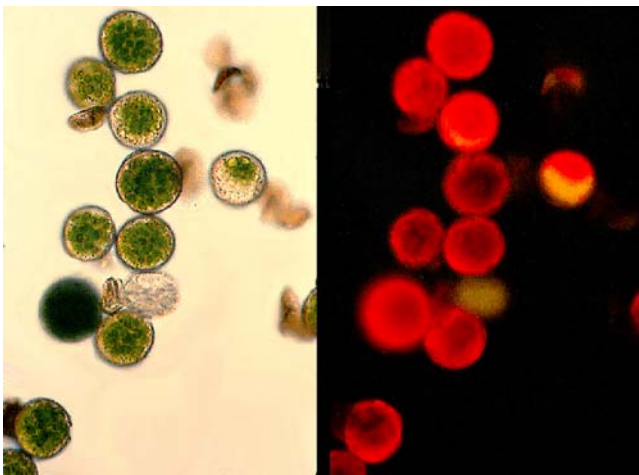


Figure 85. Spores of *Fontinalis squamosa* showing spores in white light on left and fluorescing under UV light on right. Note that the living spores show up as red under fluorescence, whereas dying and dead spores are yellow or invisible. Photos by Janice Glime.



Figure 86. *Timmiella barbuloidea*, 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 [DiOC₆(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 $\mu\text{g ml}^{-1}$ are used, whereas to see Basidiomycetes that form endophytic associations, the concentration needs to be at least 50 $\mu\text{g ml}^{-1}$. 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 decolorized

FAA (Formalin-Acetic-Alcohol) (100 ml)

Ethyl alcohol	50 ml
Glacial acetic acid	5 ml
Formaldehyde (37-40%)	10 ml
Distilled H ₂ O	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.

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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*.

Stain	Internal Layer		Epidermal Layer	
	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.

pH Testing (Zander 1980; Long 1982)

Lichenologists are quite familiar with testing pH reactions, but this technique has not been widely used on bryophytes. Zander (1980) used pH 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 H₂SO₄, 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 pH 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 pH changes. Photo by David T. Holyoak, with permission.



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

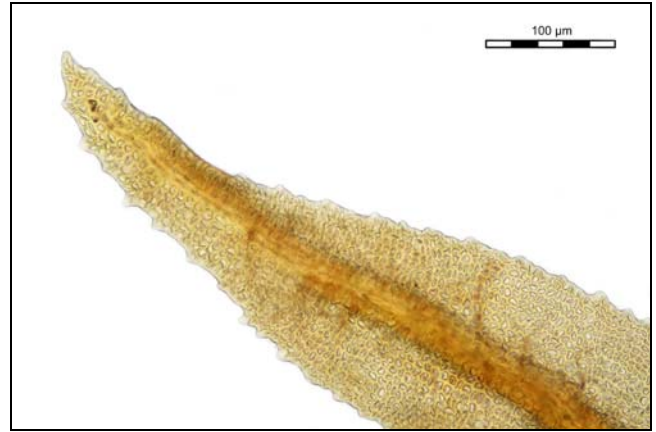


Figure 92. *Bryoerythrophyllum wallichii* leaf cells, a species that changes color ranging from pale brown to dark red-brown in response to decreasing pH. Photo from Trustees of the Natural history Museum, London, through Creative Commons.



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



Figure 94. *Leptodontium flexifolium*, a species that may be green, orange, red, or brown, depending on the pH. 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 pH. 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 pH ~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 pH ~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 pH 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.

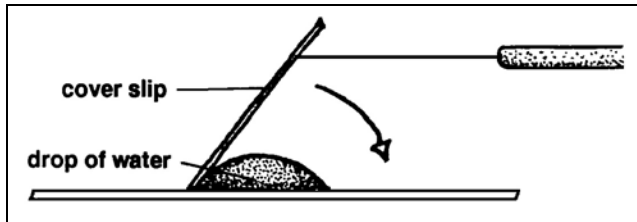


Figure 99. Technique for making a slide with minimal air bubbles. Drawing by Janice Glime.

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.

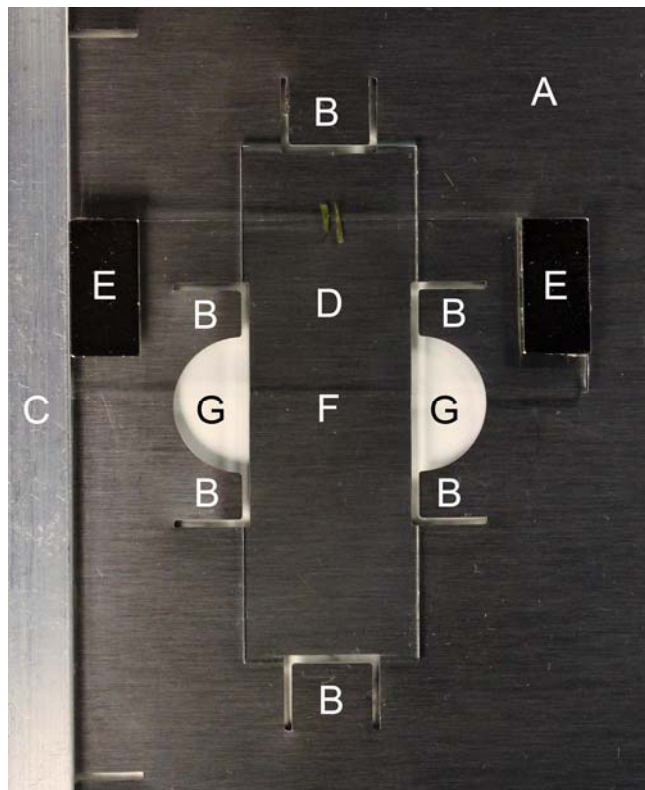


Figure 101. A magnetic sectioning aid with fixed *Polytrichum* leaves ready for sectioning. The support plate (A) is a magnetic, rust-free steel sheet with laser cut tongues (B) which are slightly bent upwards, and a non-magnetic strip (C) which helps to align the magnetic slide (D) with its attached 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.

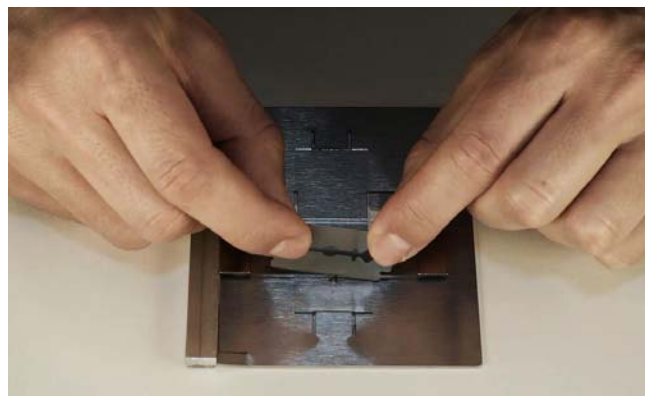


Figure 102. Sectioning with razor blade and magnetic slide holder. Photo courtesy of Daniel Hepenstrick.



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.
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.

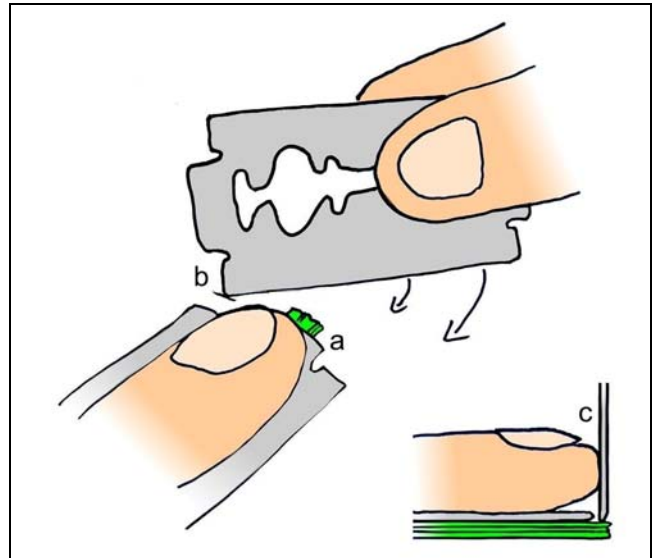


Figure 104. Lüth leaf sectioning. The bryophyte is green, shown at **a** and in the side view at **c**. The cutting position of the double-edge razor blade is at **b**. Photo courtesy of Steve Rae.

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.

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.

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."

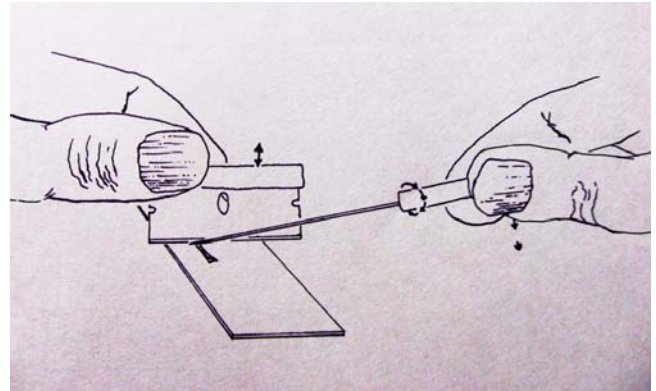


Figure 106. The roll-and-chop method of sectioning bryophytes. This would usually be done while looking through a dissecting microscope. Modified from Wilson (1990).

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 \times 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.

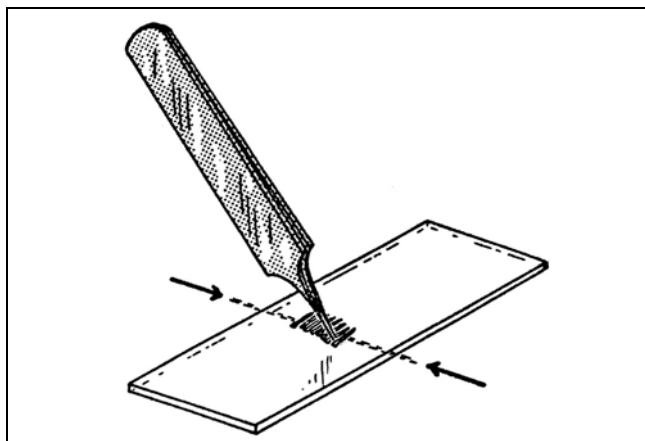


Figure 109. Placing specimen on slide in first step of the double slide sectioning technique of Sean Edwards. Drawing by Sean Edwards.

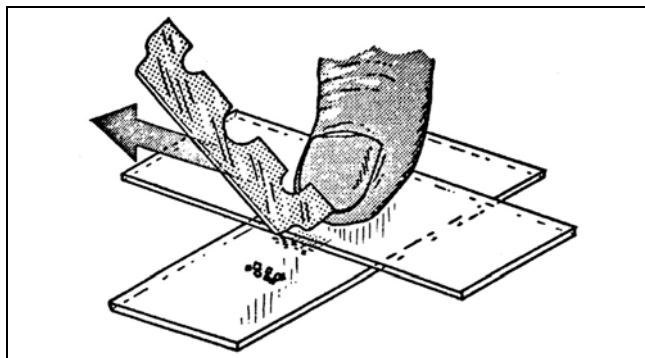


Figure 110. Alignment of slide and specimens in double slide sectioning technique of Sean Edwards. Drawing by Sean Edwards.



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

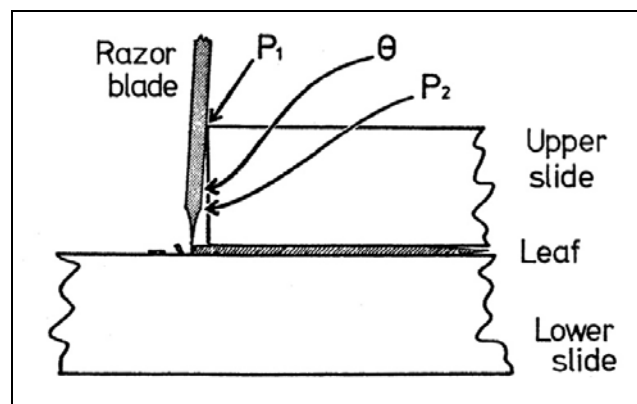


Figure 112. Cutting position of the razor blade in the double slide sectioning technique of Sean Edwards. θ is the angle of tilt and P_1 is the fulcrum. P_2 is the position of the fulcrum after the tilt of the blade has passed 0° (vertical). Drawing by Sean Edwards.

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.

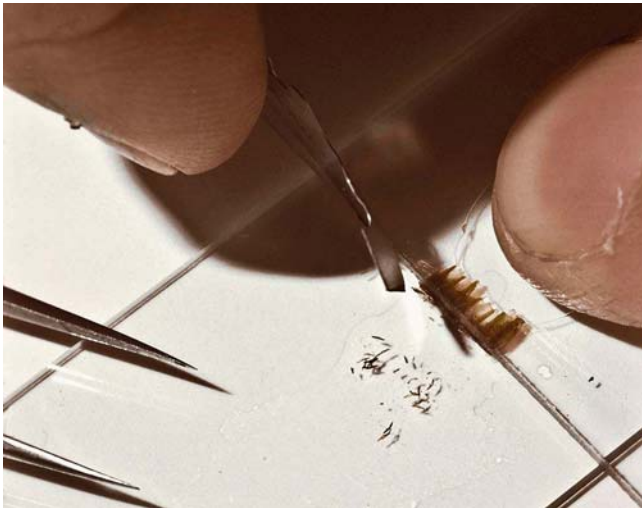


Figure 114. Cut sections along edge of top slide in double slide sectioning technique of Sean Edwards. Note the alignment of multiple stem pieces under top slide. Photo by Sean Edwards.

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 $\sim 45^\circ$ 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 $\sim 30^\circ$ 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 $\sim 30^\circ$ 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.

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.)



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

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.

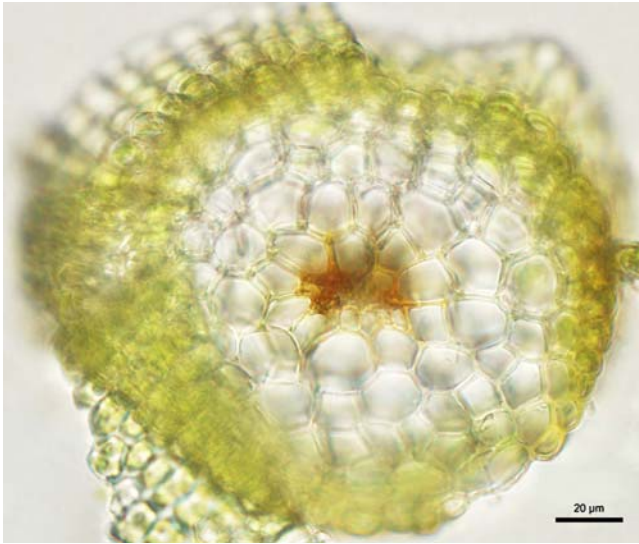


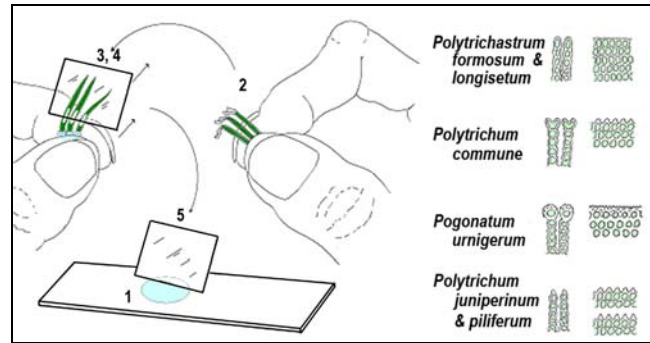
Figure 116. Stem cross section of the moss *Molendia sendtneriana* showing central strand. Photo from Dale A. Zimmerman Herbarium, Western New Mexico University, with permission.

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).

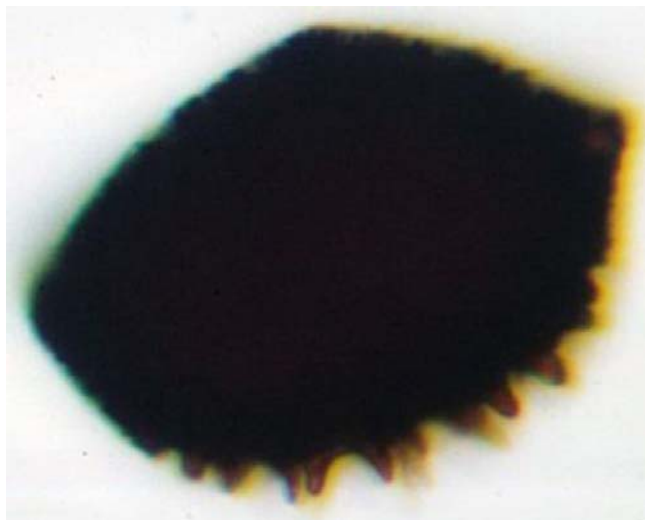


Figure 117. Spore of *Riccia sorocarpa* showing its dark color and density, preventing one from seeing spore wall details without special techniques. Photo from EOL through Creative Commons.

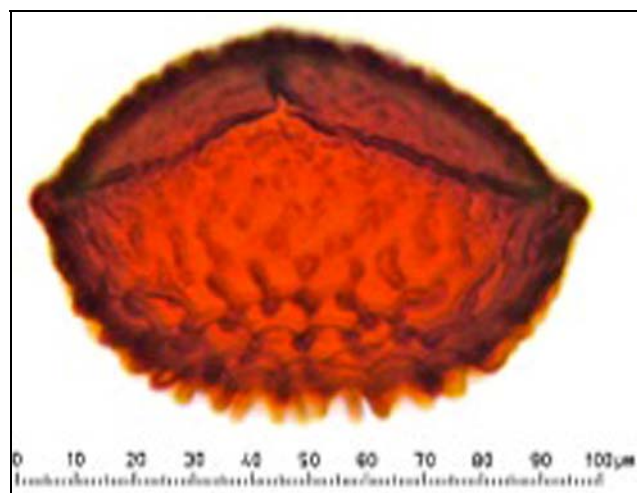


Figure 118. Spore of *Riccia sorocarpa* using both transmitted and reflected light plus stacking. Compare the clarity to that of the same species in Figure 117. Photo by David Wagner, with permission.

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** ($\text{Ca}(\text{ClO})_2$), 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 *Ricciocarpus 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!

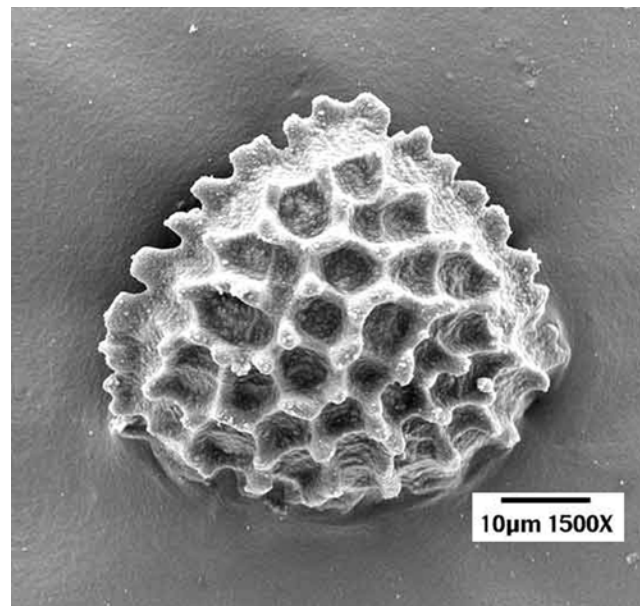


Figure 119. Distal SEM view of *Ricciocarpus natans* spore. Photo by William T. Doyle, with permission.

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.

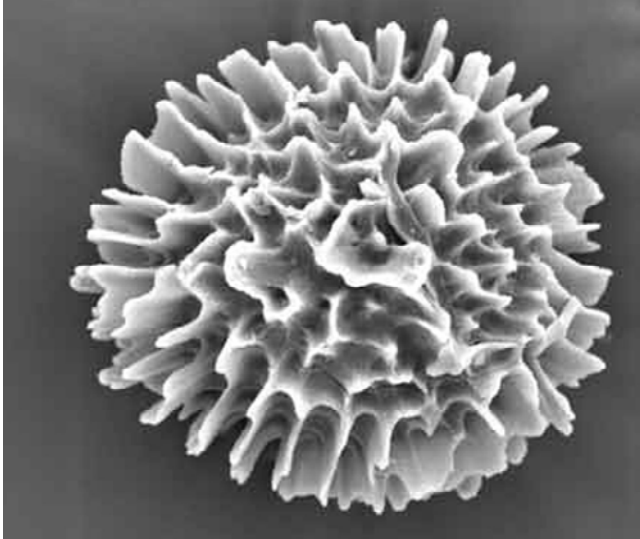


Figure 120. *Fossombronia longiseta* spore proximal SEM. Photo courtesy of William T. Doyle.

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.

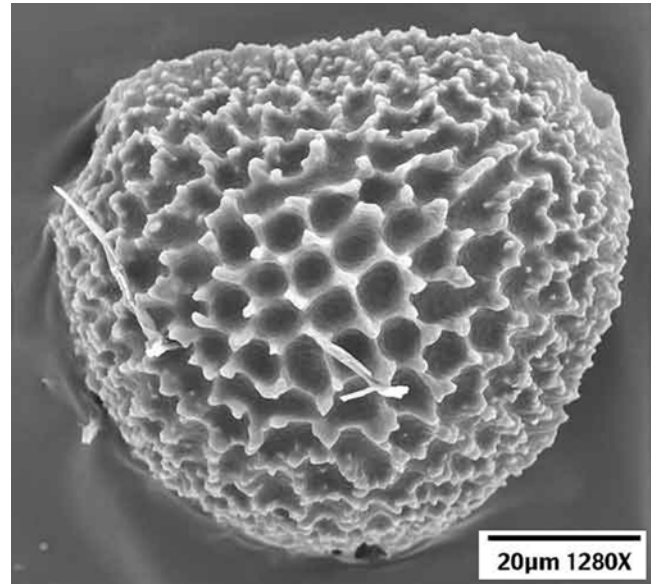


Figure 121. *Riccia sorocarpa* distal spore wall SEM. Photo courtesy of William T. Doyle.

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.

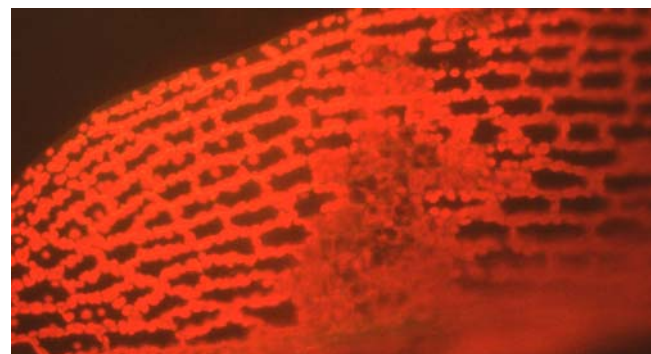


Figure 122. Leaf of *Funaria hygrometrica* showing chlorophyll fluorescence and demonstrating the clustering of chloroplasts at the cell margins. Such positioning indicates the presence of a vacuole. Photo by Janice Glime.

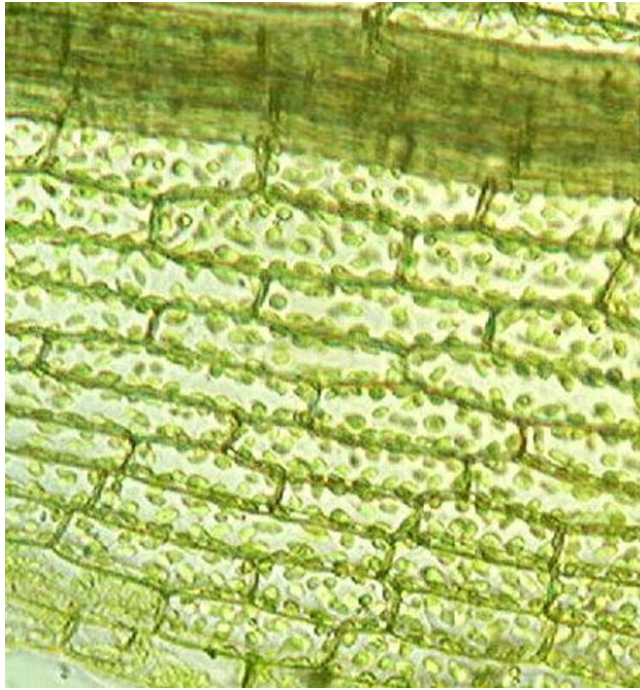


Figure 123. *Funaria flavicans* leaf cells showing chloroplasts on cell margins, indicating presence of a cell vacuole. Photo by Frederick B. Essig, with permission.

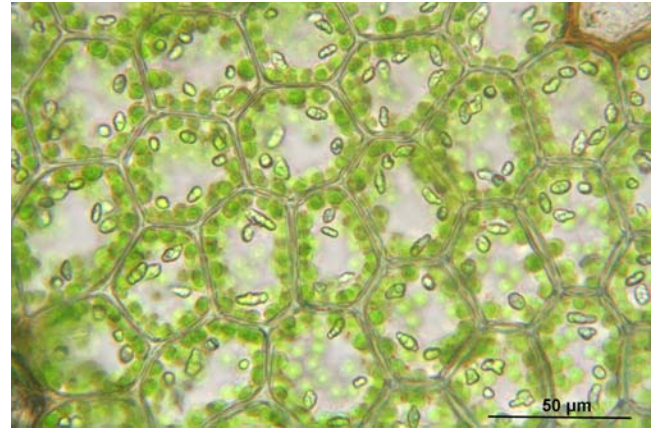


Figure 124. *Calypogeia muelleriana* leaf cells showing oil bodies. Photo by Hermann Schachner, through Creative Commons.

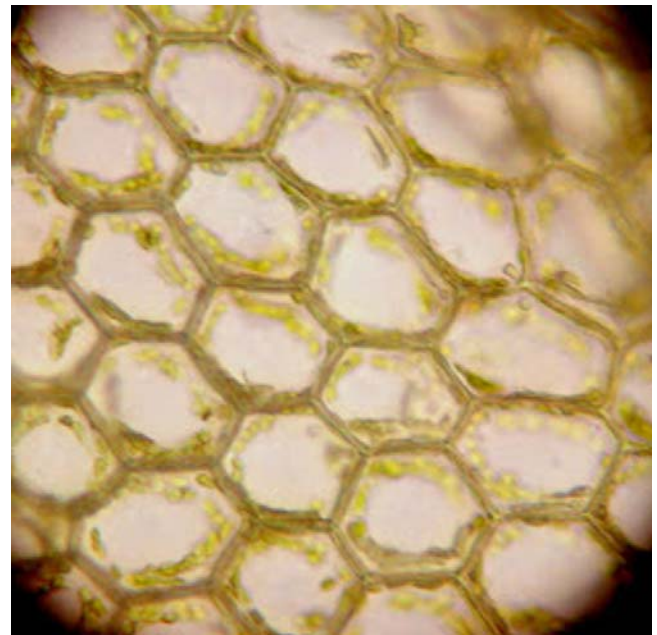


Figure 125. *Calypogeia muelleriana* dried leaf cells where oil bodies have disintegrated. Photo by Jutta Kapfer, with permission.

Liverworts and Oil Bodies

Oil bodies can be a problem because they disappear as the liverwort dries (Tom Thekathyl, 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.

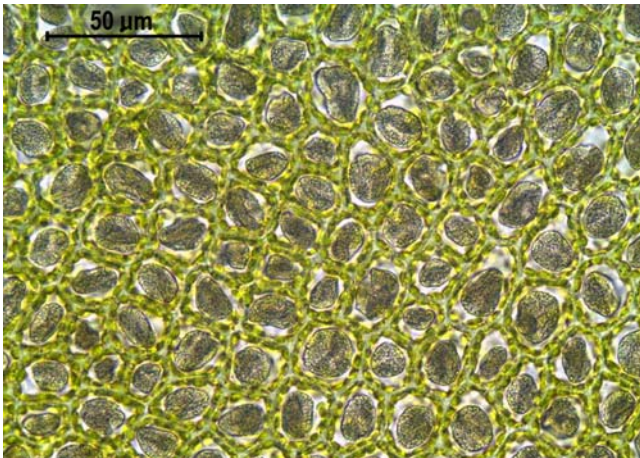


Figure 128. *Scapania gymnostomophila* leaf cells showing oil bodies. Photo by Hugues Tinguy, with permission.

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 dehiscent 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.

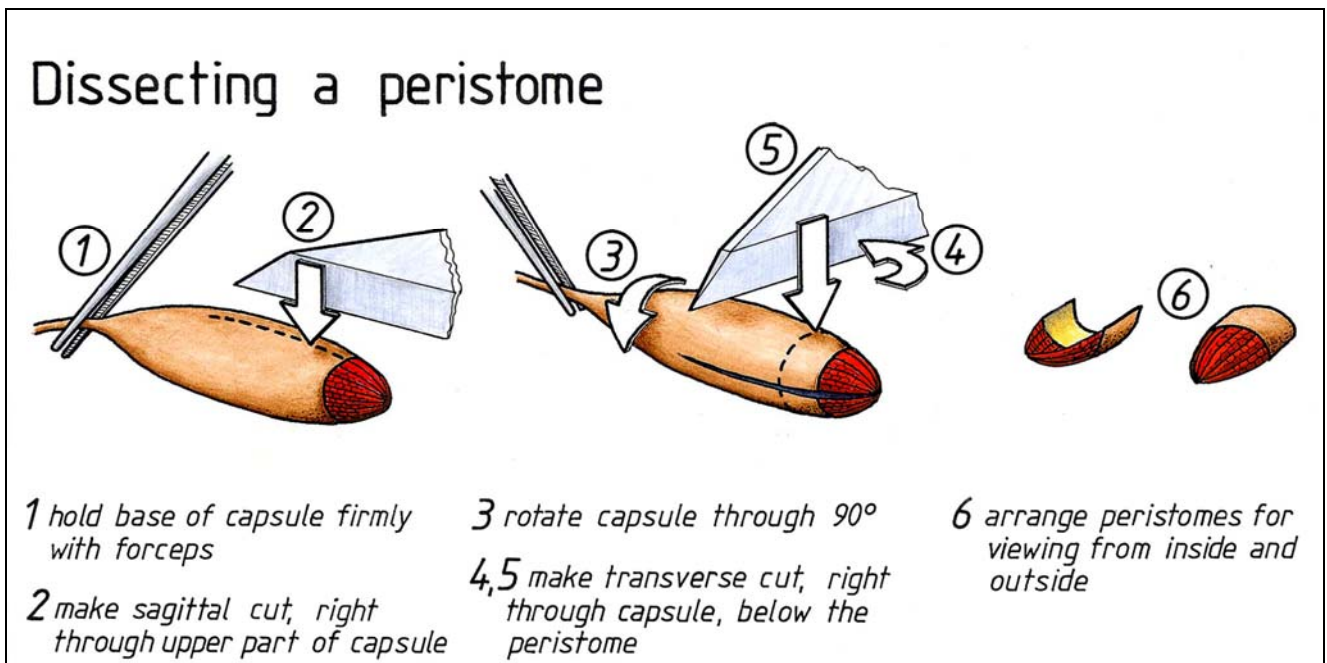


Figure 129. Cutting method for capsule to display peristome teeth on a slide. Drawings by Sean Edwards.

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*.

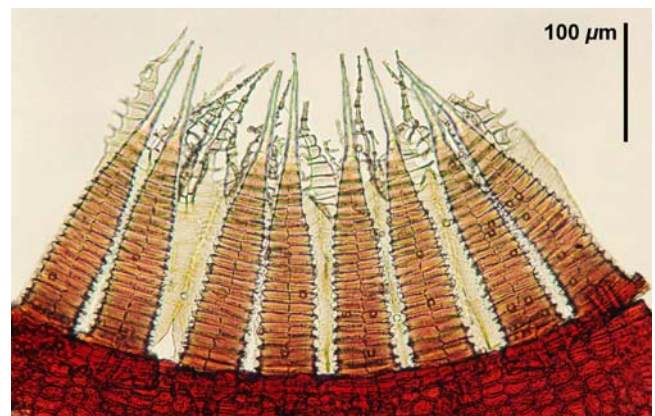


Figure 130. Peristome teeth of *Bryum dichotomum*. Photo by Sean Edwards.

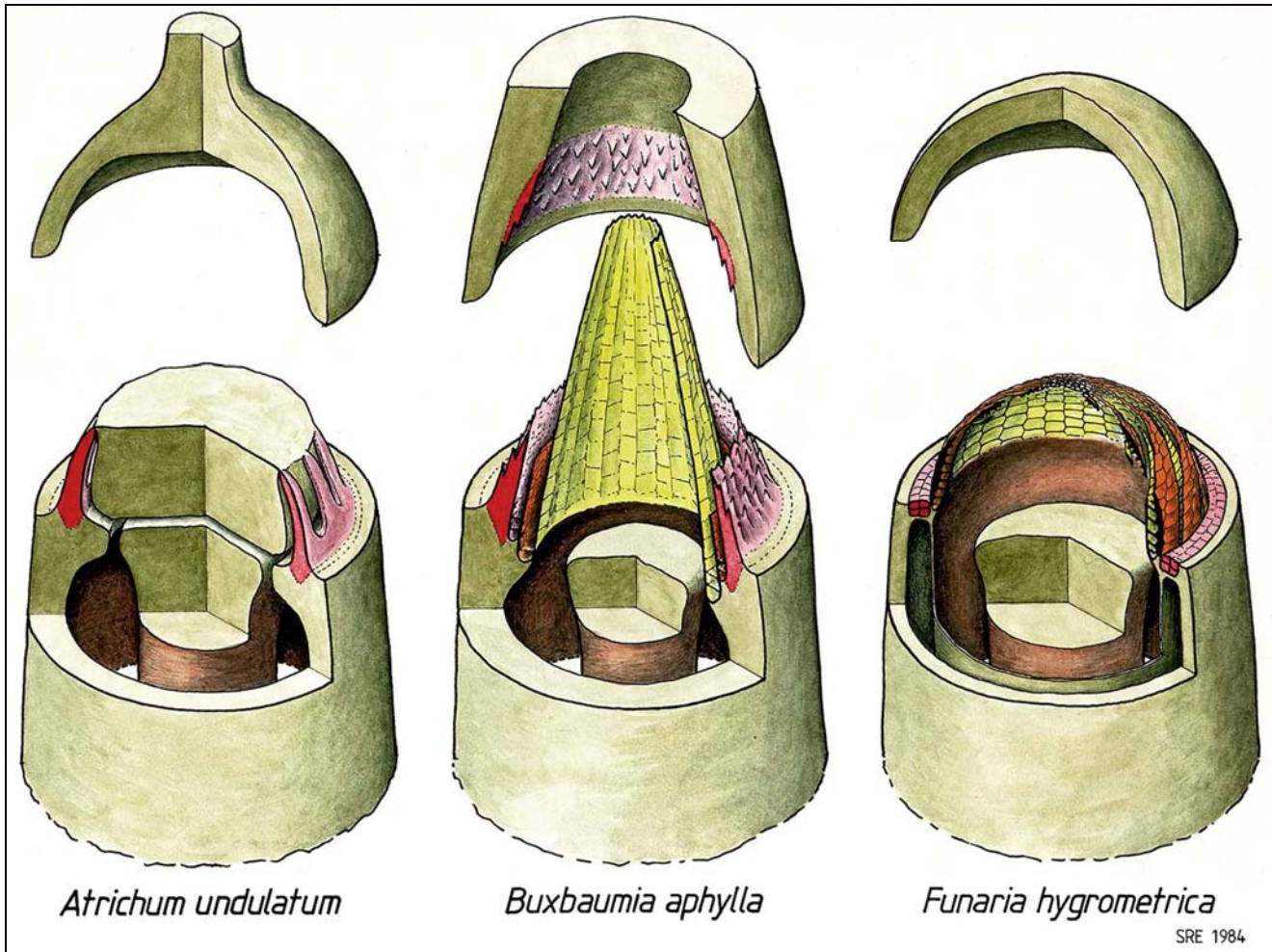


Figure 131. Three species with the operculum removed to reveal differences in peristome types. Drawings by Sean Edwards.

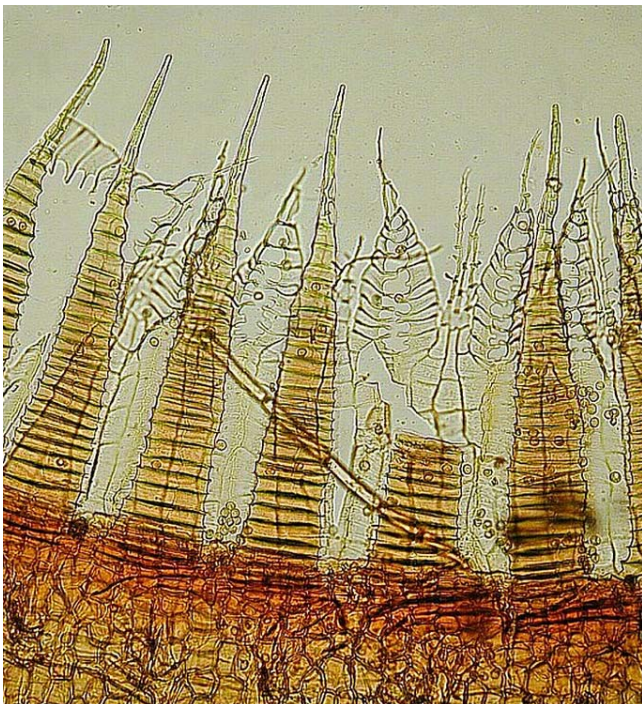


Figure 132. *Bryum caespiticium* peristome showing peristome teeth and cilia. Photo by Michael Lüth, with permission.



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, H₂O₂, 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 H₂NO₃, and H₂SO₄. Some *Sphagnum* subgenera respond to pH and have distinctive colors in NaHCO₃.

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 Bryonettters 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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