CHAPTER 2-3
LABORATORY TECHNIQUES: MAKING OBSERVATIONS

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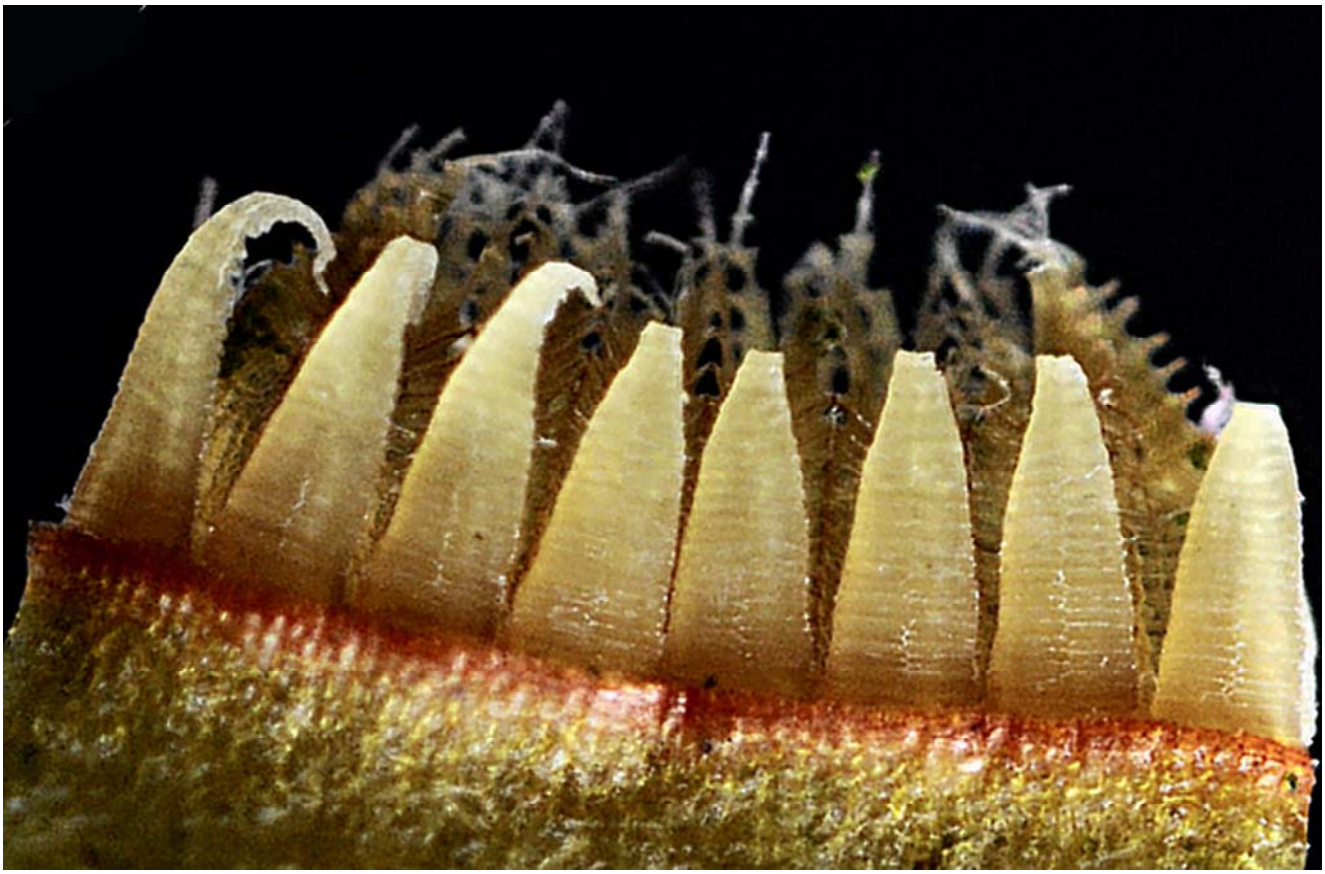

Figure 1. ***Plagiomnium rhynchophorum*** peristome ready to perform its dispersal of spores. Photo by George Shepherd through Flickr.

Sporophytes

Stomata

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

Opening Immature Capsules (Lauridsen 1972)

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

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

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

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

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

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

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

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

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

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

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

Peristomes

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

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

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

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

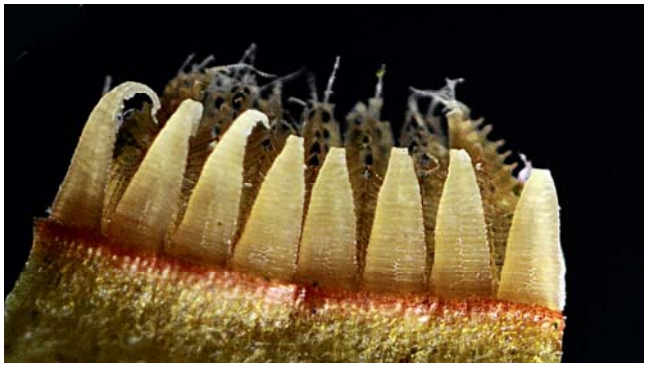

Figure 9. ***Plagiomnium rhynchophorum*** (**Mniaceae**) peristome – one that flexes in response to humidity in one's breath. Photo by George Shepherd, with permission.

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

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

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

Anchoring Specimens in Clay

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

Counting Spores

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Spore Dispersal

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

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

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

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

Sperm

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

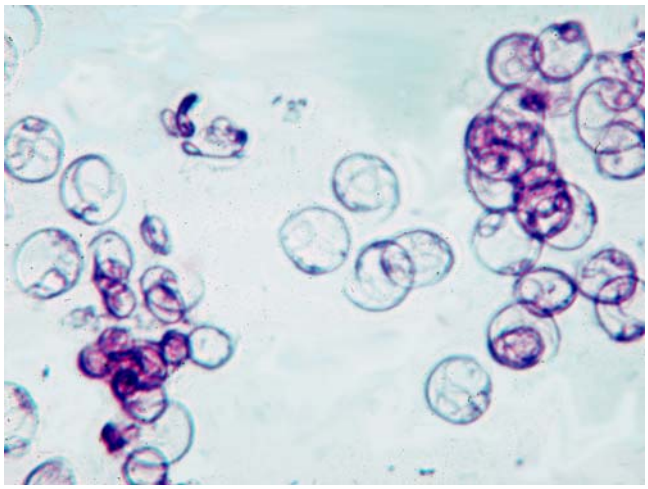

Figure 12. Stained bryophyte sperm. Image modified by Janice Glime.

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

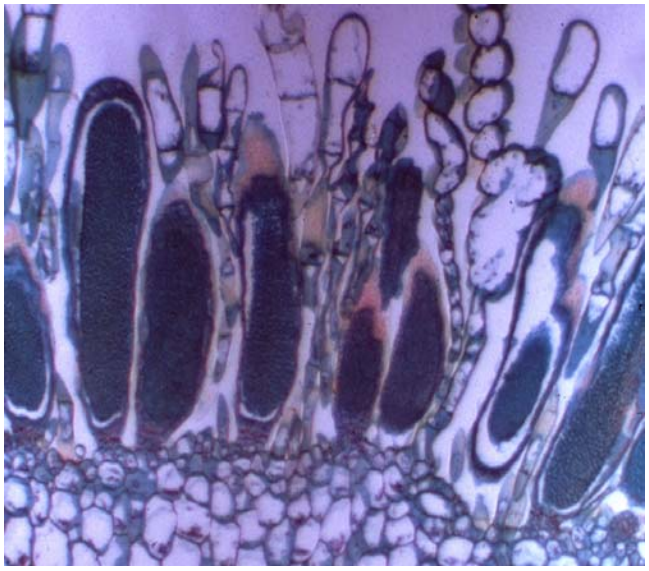

Figure 14. ***Polytrichum*** antheridia, where sperm are produced. Photo by Janice Glime.

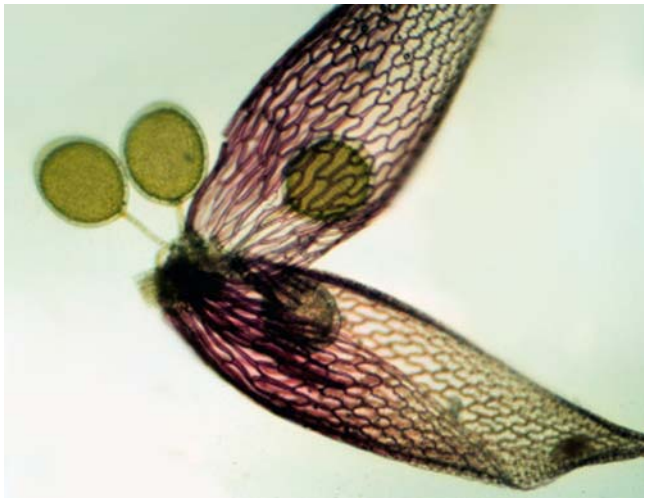

Figure 15. ***Sphagnum*** antheridia; these release sperm in autumn. Photo courtesy of Yenhung Li.

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

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

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

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

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

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

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

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

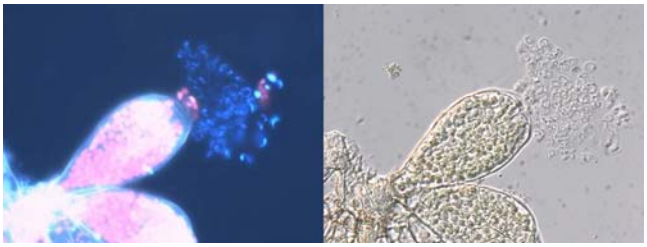

Figure 17. Sperm with DAPI stain (**left**) compared to fresh material (**right**). Photos courtesy of Nelly Horst.

Paraphyllia

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

Axillary Hairs

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

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

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

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

Leaf Movement

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

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

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

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

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

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

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

Water Movement

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

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

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

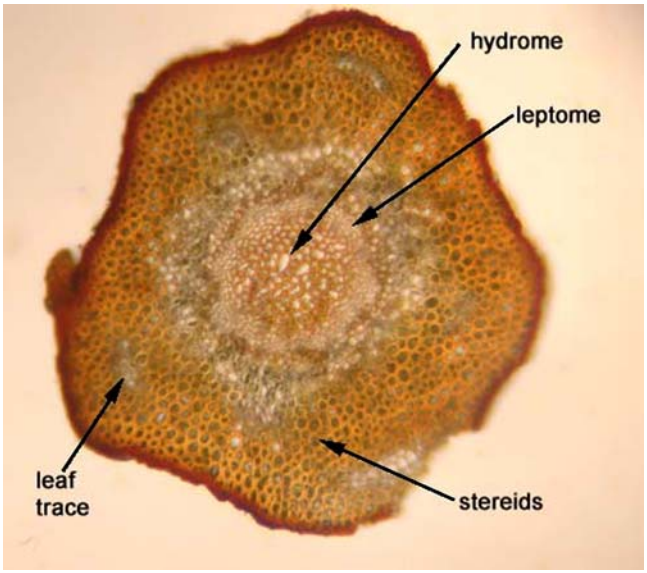

Figure 24. ***Polytrichum commune*** stem cross section showing leaf traces. Photo from UBC botany website, with permission.

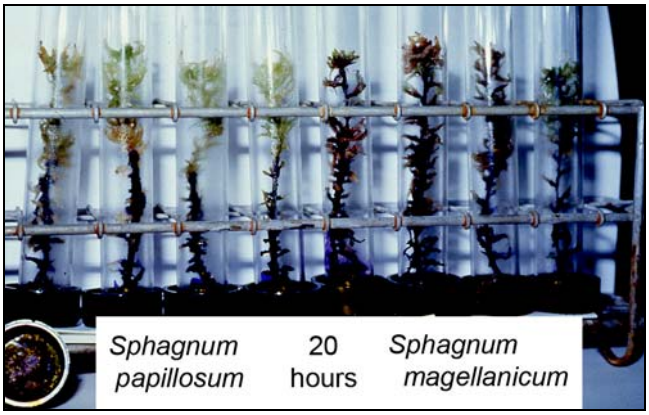

Figure 25. Demonstration of external water conduction in two species of ***Sphagnum***. Note that it has travelled much farther in ***Sphagnum magellanicum*** than in ***S. papillosum***. Photo by Yenhung Li.

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

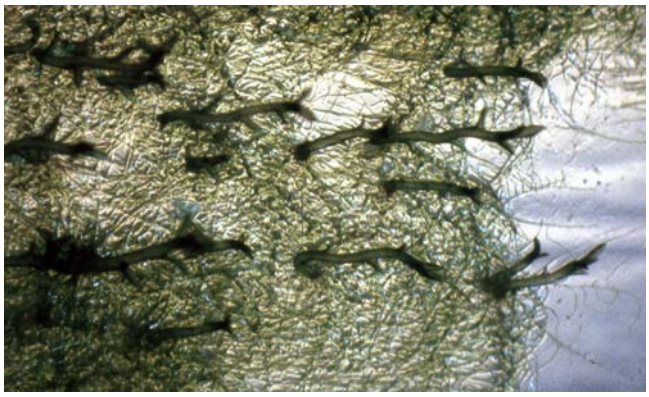

Figure 26. Phototropism of ***Funaria hygrometrica*** with light coming from right side of Petri plate. Photo by Janice Glime.

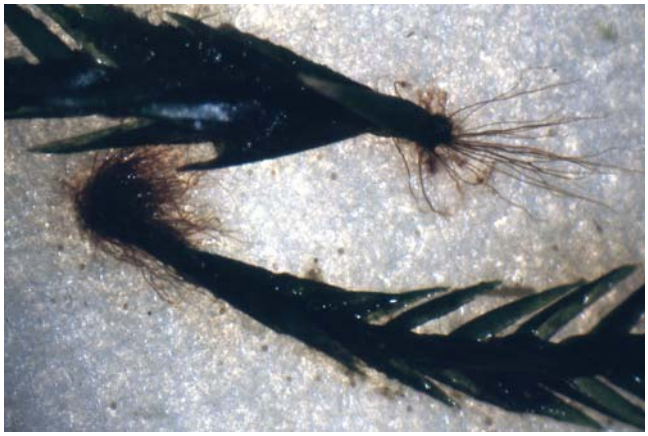

Figure 27. ***Fontinalis squamosa*** rhizoids exhibiting negative phototropisms to light coming from the left. Photo by Janice Glime.

Tropisms

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

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

Etiolation

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

Splash Cup Dispersal

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

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

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

Determining Oicy

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

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

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

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

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

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

Brownian Movement

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

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

Plasmolysis

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

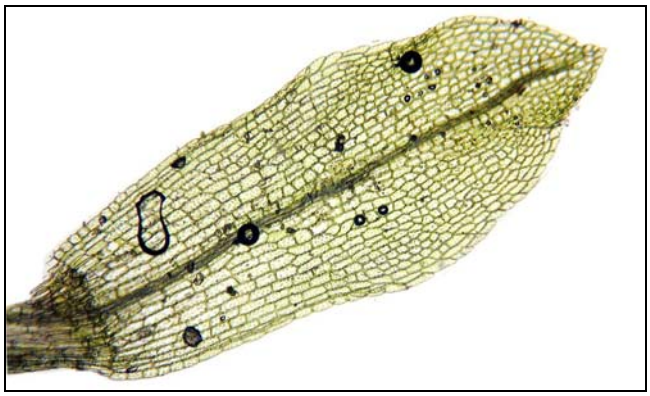

Figure 34. ***Physcomitrium sphaericum*** leaf cells, a good species for demonstrating plasmolysis. Photo by Michael Lüth, with permission.

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

Nutrient Cycling

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

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

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

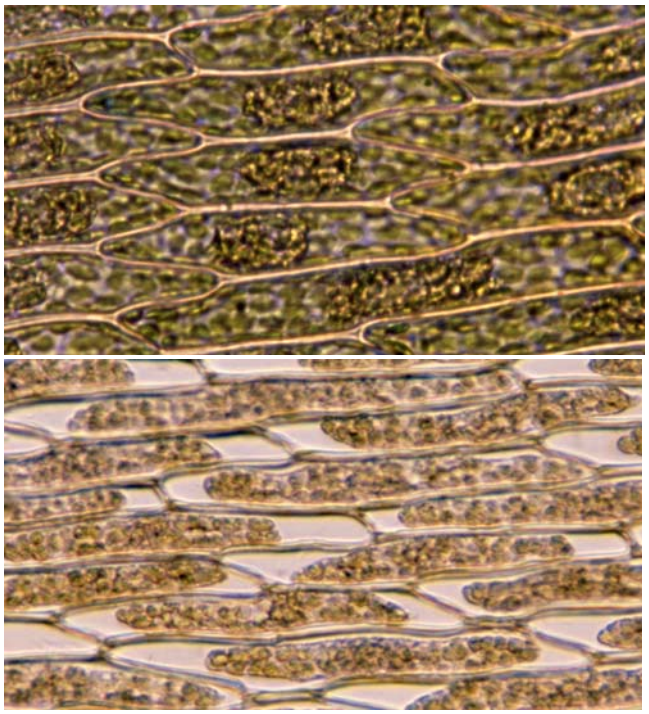

Figure 36. ***Fontinalis duriaei*** leaf. **Upper:** healthy leaf cells in water, demonstrating normal cell protoplasm arrangement. **Lower:** ***Fontinalis duriaei*** leaf cells in 100 µg L^{-1} copper, showing plasmolysis of cell contents.

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

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

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

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

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

Measuring

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

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

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

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

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

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

Calibrate

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

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

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

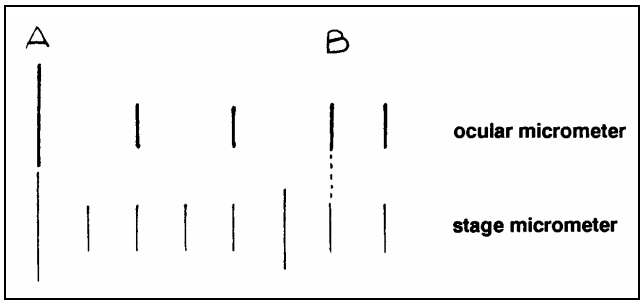

Figure 40. Alignment of scale of ocular micrometer (reticule) with that of the stage micrometer as seen in the eyepiece of the microscope. Drawn by Shelly Meston.

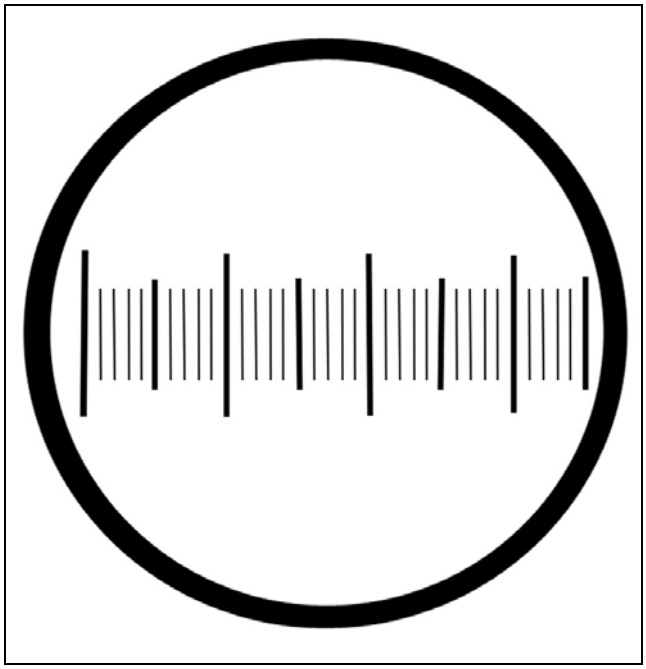

Figure 41. View of an ocular micrometer (reticule) in the eyepiece of the microscope. Drawn by Janice Glime.

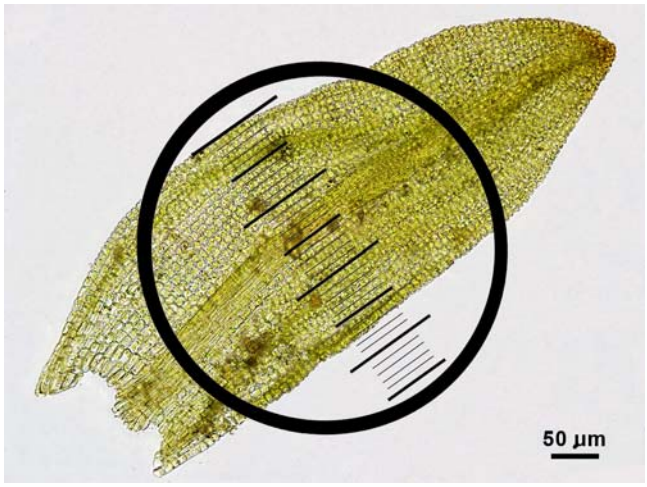

Figure 42. Reticule in position to measure width of midleaf of ***Barbula convoluta***. Measured width is 28 units and must be calibrated against stage micrometer. Leaf image by Dale A. Zimmerman; reticule by Janice Glime.

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

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

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

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

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

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

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

Leaf Measurements

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

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

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

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

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

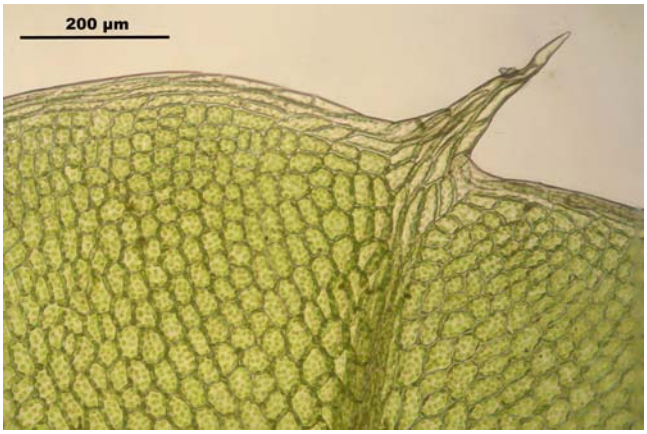

Figure 44. ***Plagiomnium elatum*** showing oblique rows of leaf cells. Photo by Hermann Schachner, through Creative Commons.

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

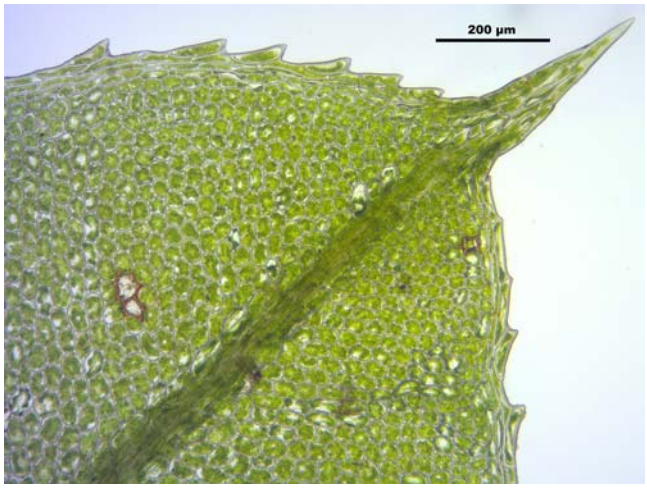

Figure 46. ***Plagiomnium medium*** demonstrating the difficulty in measuring cells in such irregular arrangements. Photo by Hermann Schachner, through Creative Commons.

Leaf Angles

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

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

Use of the Ocular Protractor

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

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

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

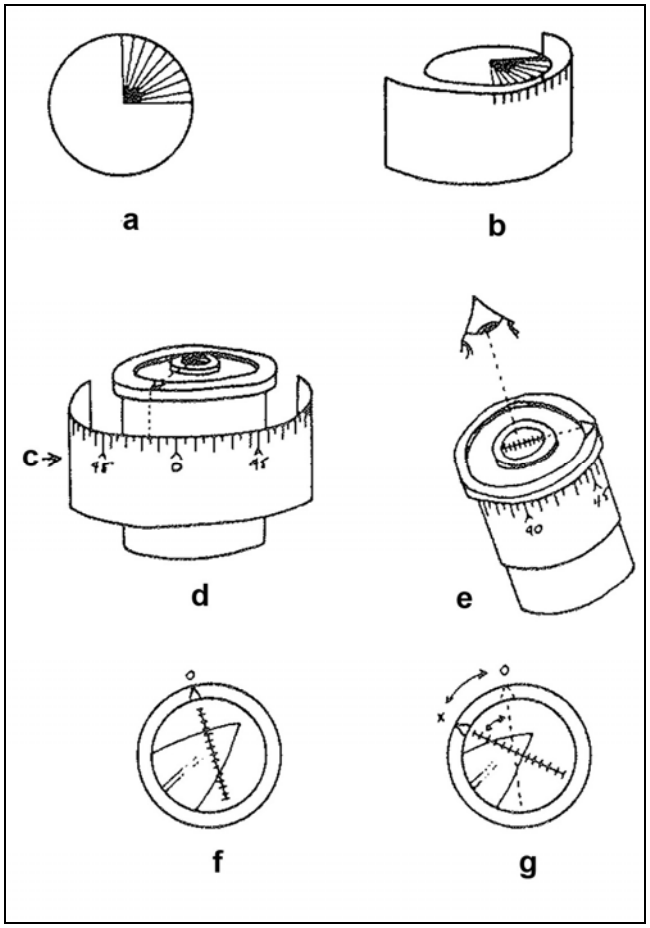

Figure 47. Ocular protractor for measuring angles. Modified from Christy 1987.

Rigidity Index

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

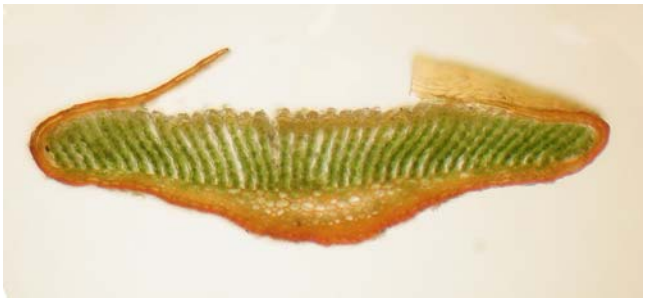

Figure 48. ***Polytrichum juniperinum*** leaf cross section showing vertical lamellae and width of leaf lamina. Photo courtesy of John Hribljan.

Making Drawings

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

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

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

Summary

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

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

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

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

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

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

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

Acknowledgments

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

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