# CHAPTER 2-3
## LABORATORY TECHNIQUES: MAKING OBSERVATIONS

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CHAPTER 2-3
LABORATORY TECHNIQUES:
MAKING OBSERVATIONS

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

**Sporophytes**

**Stomata**

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

**Opening Immature Capsules (Lauridsen 1972)**

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

Although the method did not work well with *Funaria hygrometrica* (14% opened), it was more than 90% successful in *Bryum argenteum*, *B. inclinatum*, *B. intermedium*, *Mnium hornum*, and *Polytrichum commune* (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.

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 Pohlstoiffe for 15-20 minutes, or longer. To avoid precipitation that occurs with Pohlstoiffe 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.

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 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.

I have had success in observing peristome movement with Dicranella heteromalla 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 to gyrate, then the peristome teeth begin to flex. Breathing on it might give the same result.

**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.

**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.

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.

**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 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 typically disperses sperm in early spring, Sphagnum in autumn (Jeff Duckett, Bryonet 11 January 2012). Pellia, which has the largest sperm, disperses in early summer. Of course if you see new growth arising from the antheridial head, you have missed the dispersal event.

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 remove the acetocarmine stain (Mogensen 1978). Living spores stain red, whereas dead spores do not stain at all. Instructions are available on their web page.

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.

**Leaf Movement**

Beginning students are often in awe when they drop water onto a moss like Hedwigia ciliata. 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 is great for a spreader; Mnium for non-spreaders (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, the large, non-chlorophyllous area at the base of the leaf 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.

**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 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 2). 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.
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.

**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 3-Figure 4). 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 3). Figure 5 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 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.

**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* 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.

**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* 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. Figure 6 demonstrates plasmolysis in *Fontinalis duriaeii*, caused by a copper solution. 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 6. *Fontinalis duriaeii* leaf. Upper: leaf in water, demonstrating normal cell arrangement. Lower: leaf in 100 µg L⁻³ copper, showing plasmolysis.](image)

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*, *Eurhynchium*, and forest floor leaf litter, for example.

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.

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 7), 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 7). 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 8-Figure 10). 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 8-Figure 10). Remember that the stage is where all your organisms will sit on slides and perform for you (Figure 10). 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).
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):

\[
\frac{\text{mm}}{\text{ocular unit}} = \frac{0.01 \times \text{# stage divisions}}{\text{# ocular divisions}}
\]

For example, in Figure 8 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:

\[
\frac{6 \times 0.01 \text{ mm}}{3} = 0.02 \text{ mm/unit or } 20 \mu\text{m}.
\]

Figure 8. 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 9. View of an ocular micrometer (reticule) in the eyepiece of the microscope. Drawn by Janice Glime.

Figure 10. 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.
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.

<table>
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<tr>
<th>Power</th>
<th>Stage Micrometer Units</th>
<th>Ocular Micrometer Units</th>
<th>mm/unit</th>
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<tr>
<td>Low power</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Medium power</td>
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<td></td>
</tr>
<tr>
<td>High power</td>
<td></td>
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<td></td>
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<td>Oil immersion</td>
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**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 11, 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 11, 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 11, 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 11, 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 11, 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 11, 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 11, 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 11](image-url) Ocular protractor for measuring angles. Modified from Christy 1987.
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.

Literature Cited


