CHAPTER 2-4
LABORATORY TECHNIQUES: PRESERVATION AND PERMANENT MOUNTS

Janice M. Glime and David M. Wagner

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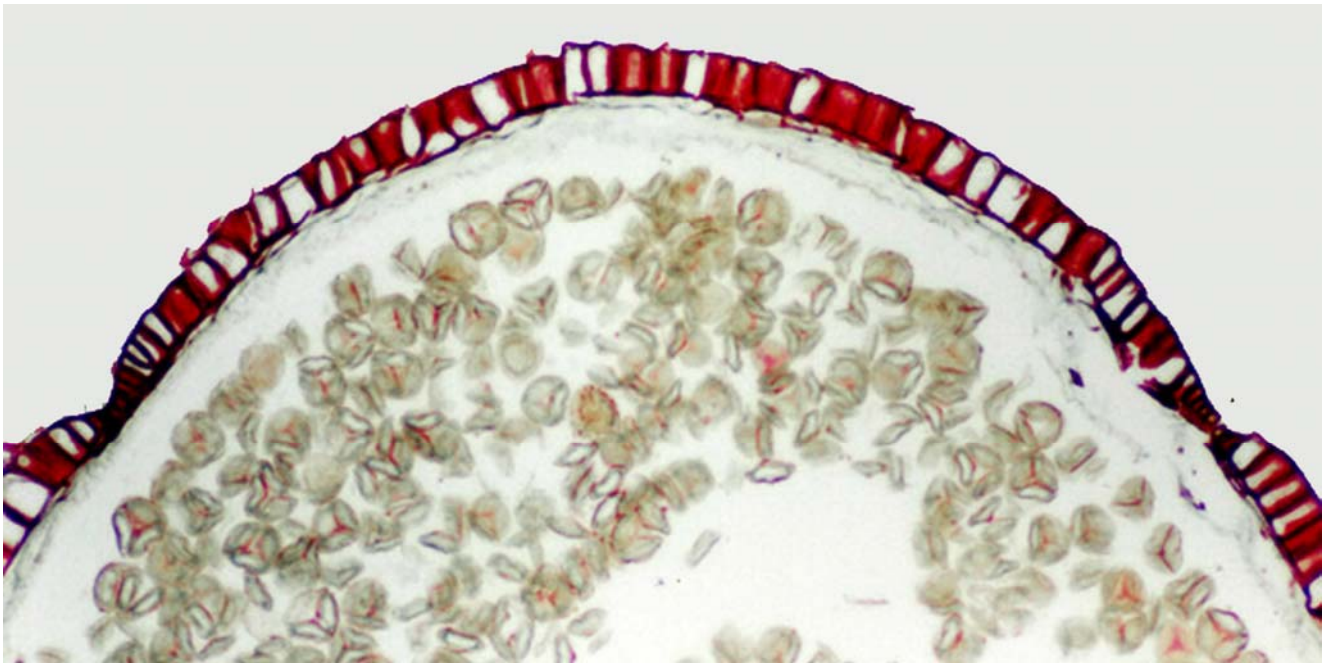


Figure 1. Stained permanent mount of ***Sphagnum*** capsule from Triarch. Photo by Janice Glime.

Permanent and Semi-Permanent Slides: Mounting Media – Mountants

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

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

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

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

Glycerine to Gum Arabic

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

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

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

Hoyer's Solution

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

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

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

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

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

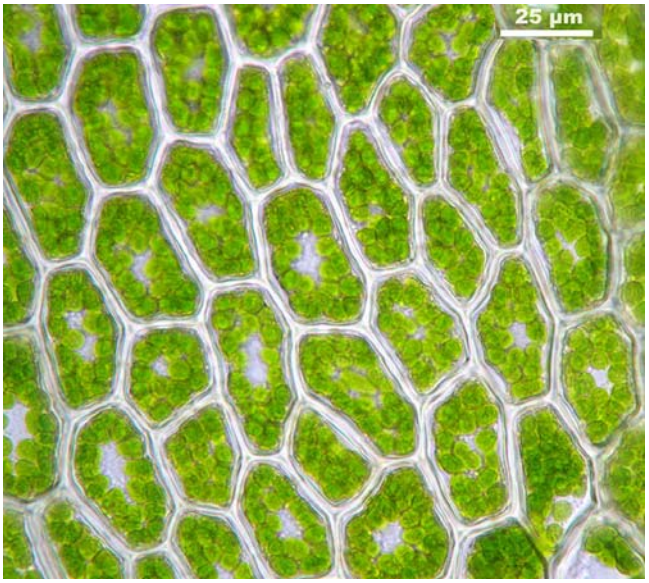


Figure 3. ***Mnium spinosum*** leaf cells. Cells of members if this family (Mniaceae) exhibit cell shrinkage and distortion in Hoyer's solution. Photo by Ralf Wagner <www.dr-ralf-wagner.de>.

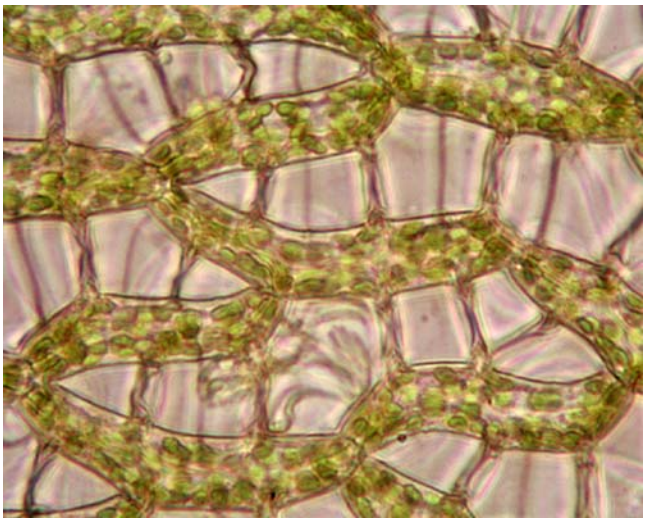


Figure 4. ***Sphagnum palustre*** cells. Cells in **Sphagnaceae** are able to retain their shape in Hoyer's solution. Photo by Malcolm Storey through Creative Commons.

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

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

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

1. Mix and stir well.

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

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

Modified Hoyer's for Chromosomes

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

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

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

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

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

Gum Chloral Solution

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Glycerine, Glycerol, and Glycerine Jelly

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

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

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

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

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

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

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

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

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

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

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

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

Glycerine Jelly Preparation (Zander 2003)

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

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

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

Using Glycerine Jelly

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

Making Semi-permanent Mount

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

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

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

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

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

Clearing

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

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

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

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

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

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

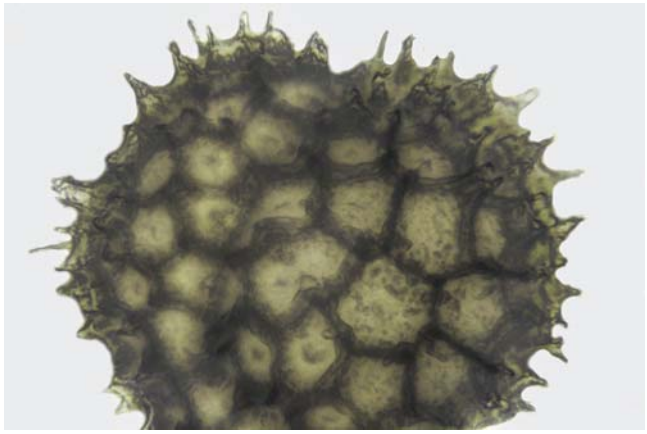


Figure 10. ***Sphaerocarpos michelii*** spore, a structure that clears well in Frahm's solution of glycerine, water, and mucilage in equal parts. Photo by Dick Haaksma, with permission.

DMHF (5,5-dimethyl Hydantoin Formaldehyde)

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

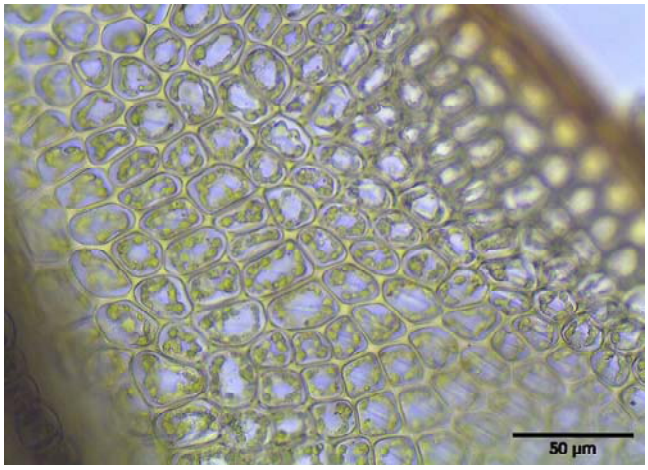


Figure 11. ***Tortula subulata*** leaf lamina cells, member of a genus wherein mountants may collapse the laminal cells. Photo by Kristian Peters.

PVA

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

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

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

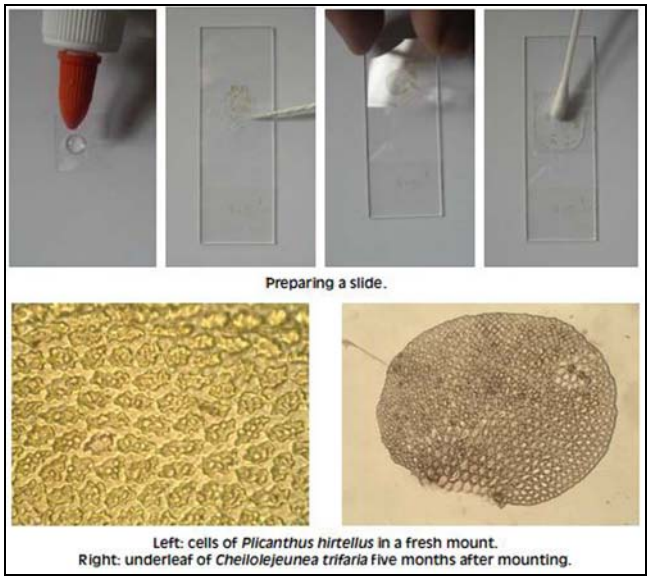


Figure 12. Meagher permanent mounting methods using glue. Photos by David Meagher, with permission.

Karo Syrup

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

Polyvinyl Lactophenol

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

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

Procedure:

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

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

Aquamount Improved

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

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

Kleermount, Xylene solution #2

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

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

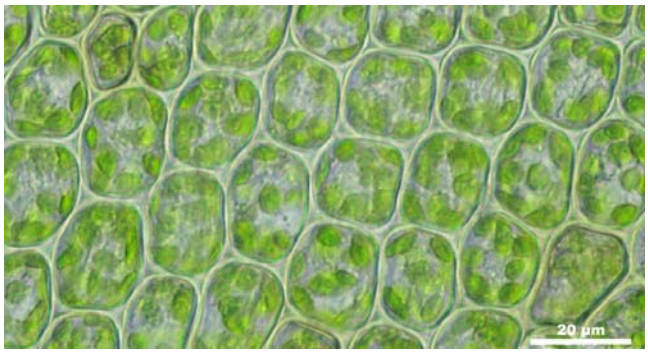


Figure 14. ***Plagiomnium undulatum*** leaf cells. This is a member of a genus in which leaf cells plasmolyze in Kleermount. Photo by Ralf Wagner <www.dr-ralf-wagner.de>.

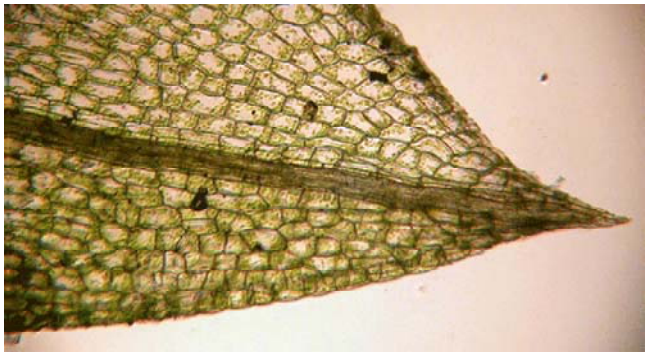


Figure 15. ***Funaria hygrometrica*** leaf cells. This is a member of a genus in which leaf cells plasmolyze in Kleermount. Photo by Bob Klips.

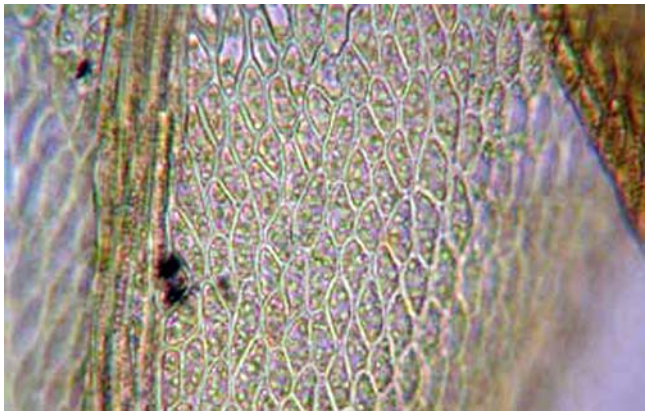


Figure 16. ***Amblystegium varium*** leaf cells. This is a member of a genus in which leaf cells plasmolyze in Kleermount. Photo by Bob Klips.

Fluoromount-G

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

Gray-Wess Mountant

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

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

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

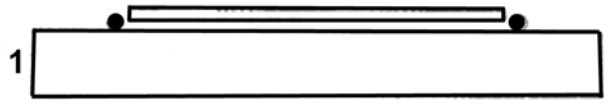
Double-Coverslip Method

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

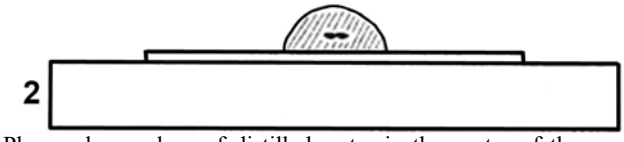
Figure 17. Ascospores of a fungus, using a double coverslip, with appendages perfectly preserved after 29 years. Photographed using Nomarski interference contrast and quartz filter. Photo from-Kohlmeyer & Kohlmeyer 1996.

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

Double Coverslip Method of Kohlmeyer and Kohlmeyer:



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

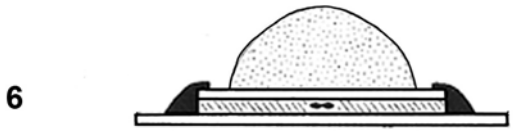


Place a larger drop of distilled water in the center of the cover glass. Add the specimen to the drop.

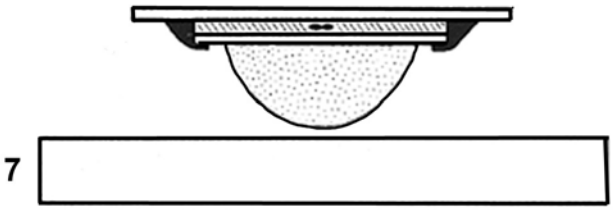
Use the smaller coverslip (18 x 18 mm) to cover the drop with the specimen, permitting immediate viewing of the living material with a compound microscope. Immersion oil can be used if necessary, but it is messy and must be cleaned off, endangering what has been accomplished. It is better to save it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

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

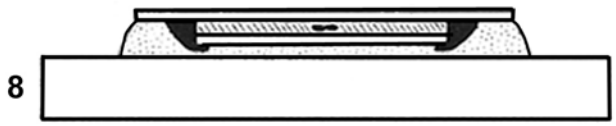
Seal the mount with a thin ring of clear fingernail polish. It is best to repeat this step after an hour to be sure the glycerine is perfectly sealed.



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



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



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

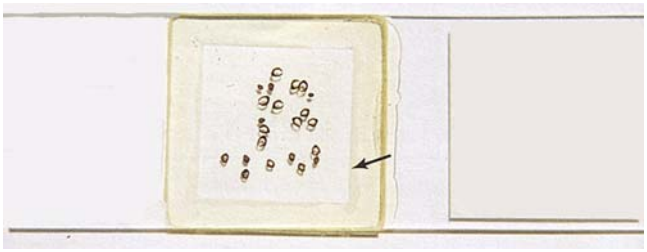


Figure 18. Double-coverslip slide showing smaller top coverslip (arrow) and nail polish seal. Note the blank label on the right. Paper labels can easily come off, so using a slide with frosted glass on one end may be a preferred solution. Labelling should be done with waterproof ink and a Rapidograph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

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

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

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

Lutants – Sealing Slides

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

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

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

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

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

Reviving Dried Slides

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

Cleaning Slides

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

Labels

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

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

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

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

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

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

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

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

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

Slide Storage

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

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

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

Preserving Bryophyte Plants for Dioramas

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

Field Collections

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

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

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

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

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

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

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

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

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

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

Preserving Liverworts

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

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

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

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

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

or

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

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

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

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

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

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

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

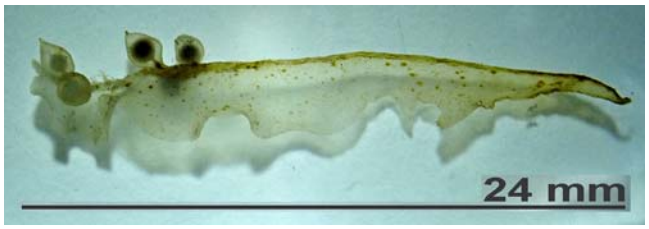


Figure 25. ***Riella americana*** with sporophytes, preserved with methanol and vinegar. Photo by David Wagner, with permission.

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

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

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

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

Summary

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

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

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

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