CrossMark

https://doi.org/10.1017/S155192950005656X Published online by Cambridge University Press

PROTOCOL OF PLANT SPECIMEN PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY (Conventional Methods)

Richard F. E. Crang
University of Illinois, Urbana-Champaign

These notes address conventional specimen preparation for higher plant tissues. While in many cases freeze-substitution has been shown to be a superior method, the equipment required is not generally available in most electron microscopic laboratories. Thus, the more conventional procedures outlined herein have been found to work satisfactorily for most leaf material, and have been most successful in the fixation and embedment of small root slices (from near the root tip). An adjustment of the osmotic pressure may be necessary if the plant material used is highly vacuolated and if there is an exceptionally high water content. The protocol contained in this communication has also been shown to produce entirely satisfactory preservations for certain yeasts (e.g., Candida, Saccharomycess) and lichens (e.g., Parmelia, Evernia, Cladonia) which are especially difficult materials to fix/embed. With such specimens, however, the fixation times are at the high end of the range.

Many plant specimens (especially leaf material) have a distinct tendency to float when placed into the initial glutaraldehyde fixative. It is generally agreed that floatation prevents fixation from being complete throughout the specimen because of the considerable difficulties in diffusion and penetration of all cellular materials. In those cases where floatation occurs, some investigators use a screen wire to hold the specimen under the surface of the fixation fluid, but this is only partially helpful in most cases due to the presence of trapped air bubbles around the specimen and even between the cells.

Thus, the best technique to "sink" the specimens is generally considered to be removal of air by vacuum treatment. Uncapped vials, about one-half full of fixative solution and with the specimens inside, may be placed inside of a vacuum oven without the heat on, or affixed to an aspiration vacuum line arranged through a water trap, and exposed to gentle vacuum for a few minutes. The better vacuum can generally be obtained with the use of the vacuum oven, and it also has a vacuum gauge attached for determining pressures more accurately. Apply the vacuum gradually until the specimens sink and small air bubbles are observed to float to the surface. If a gauge is present **carefully** hold the vacuum at ***ca.* 28** psi.

Be **very** careful to do this gradually, because too fast vacuum, or too much vacuum will result in the sudden and often violent boiling of the fixation fluid which can discharge the specimen from the liquid. Hold the samples in the immersed state for a few minutes, then admit air and repeat the process 2-3 times over the course of 20-30 mm. Many specimens will not sink until after air has been readmitted to the chamber. If only a few specimens still float, discard them. Complete the remaining steps without vacuum treatment.

There is some variation in the procedures regarding whether to perform all procedures at room temperature or to conduct some of the steps at ice bath temperature. The decision is usually based on whether the material has very high levels of hydrolytic (especially proteolytic) enzyme activity which can cause damage to the tissues faster than the fixation process. If so, then ice bath temperature should be used in **all** steps up to 95% ethanol dehydration. However, in most cases all preparative procedures can be performed at room temperature.

It is best that fixation fluids and embedding media be prepared the same day that they are used. Embedding resins should not be prepared and then stored in the refrigerator, since the cold temperature will condense moisture and deposit it into the resin. "Wet" resin will not infiltrate properly and will later appear to have many small "holes" in the sections. Store resin mixtures away from direct sunlight and in a desiccator. A vacuum desiccator, however, is not necessary for resin mixture storage.

Finally, it is important to check the pH of both glutaraldehyde and osmium fixation fluids in order to make certain that they are the same value as the buffer. In most cases, plant material fixes best at a pH value of 6.8. However, values up to 7.4 can sometimes be successfully employed, and some investigators start fixing plant tissues at a high pH (7.4 to even 8.0) and, after a short period of time (approx. 10 minutes), then drop the pH down to near neutrality. The final determination depends to a certain extent upon the kind of plant materials used, and efforts in trial-and-error.

Additional Notes:

1. Prepare Sorenson's phosphate buffer as 0.2 M stock. Combine equal parts of the buffer with 4 or 5% aqueous glutaraldehyde. It is best to work in a chemical fume hood. Glutaraldehyde from sealed ampoules is much better than that from bottles. If glutaraldehyde from bottles must be used, then make certain that the bottles have not been stored longer than 3-4 months and have been sealed under inert gas. The freshly prepared fixative should be made up in quantities not much greater than that to be used (approx. 3-4 mL/fixation vial). Check the pH at the same temperature which will be used for fixation. Carefully adjust with small drops of 0.1 N NaOH (for certain plant materials, KOH can be substituted) or 0.1 N HCl as necessary.
2. Dissect small pieces of plant material with a very sharp scalpel or dual-sided razor blade. Make sharp cuts on a rubber or waxen surface (may be Parafilm™), and be sure that specimens such as root material do not air dry in the cutting process. It is best to cut the material in a small puddle of the fixative. Cut samples approx. 2 x 2 x 1 mm in size or smaller. Do not squash or tear the material during the cutting procedure. Transfer specimens by picking them up adhering to the surface of a tapered or pointed applicator stick - not with forceps.
3. Transfer solutions by pipetting off the previous solution with a Pasteur pipette and **quickly** replacing the solution with the next one. Be **very** careful not to let the specimens dry at any time. It is preferable to leave a very small amount of the previous solution over the specimen than to remove all and allow drying to occur. It may be useful to make two changes of solution each time in order remove the first solution.
4. Prepare osmium tetroxide solution just before its use by thoroughly mixing equal volumes of 2% osmium tetroxide solution with 0.2 M phosphate buffer. This must be done in a chemical fume hood. It is best to use liquid osmium solutions from sealed ampoules. Making osmium up from granules must be done at least one day in advance of its use. Carefully adjust pH with small drops of 0.1 N NaOH (or KOH as indicated above) or 0.1 N HCl as necessary.

Schedule:

1. Fix in glutaraldehyde solution (as above) for 4 hours to overnight.
2. Make three brief (2-3 minutes each) washes with 0.2 M phosphate buffer (stock). Swirl the specimens around frequently. Do **not** allow the specimens to adhere to the side of the vial above the fluid level where air drying can occur.
3. Post-fix in osmium tetroxide solution (as above) for 2-3 hours. If room temperature fixation is used; 3-4 hours if ice bath temperature is used. Seal vial during fixation time. Work in chemical fume hood. Swirl specimens occasionally.
4. Pipette off the osmium tetroxide solution, and replace with 0.2 M phosphate buffer, making two changes of 2-3 minutes each.
5. Go through ethanol dehydration series step-by-step for 15-20 minutes at each step. Suggested steps are: 25%, 50%, 75%, 85%, 95%. If using cold dehydration, return to room temperature after 95% ethanol.
6. Complete ethanol dehydration by making 2 changes of absolute (100%) ethanol for 15 minutes each at room temperature. Purity of absolute alcohol can be better assured if Linde™ molecular sieve desiccant beads are used. **Do not use Dryerite™.**
7. Change solution to equal parts of absolute ethanol and pure propylene oxide for 15 minutes. Propylene oxide may be stored at room temp. with Linde™ molecular sieve beads. Do not allow contact with moist air. Despite vendors recommendations, it is best not to refrigerate propylene oxide in order to avoid condensation of moisture into the reagent. However, store propylene oxide in a fume hood and away from any sparks or flames.
8. Go through three changes of pure propylene oxide, each change for 10 minutes at room temperature.
9. Infiltrate with 3 parts propylene oxide, one part resin mixture (see details below on the formulation of resin mixture). Place stoppered vial(s) on rotator at room temperature for 3-4 hours. Store the samples in a dry location, but not in a completely sealed chamber where propylene oxide fumes may accumulate dangerously.

Continued on Page 20

Elite™ piezoelectric technology actively cancels floor vibration. Semi-kinematic docking stabilizes work surface during parts transfer.

The future of vibration control technology is crystal clear.

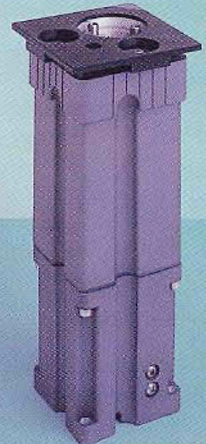
To bring you superior vibration control today, we're focusing on the technology of tomorrow.

Elite 3 active vibration cancellation workstations provide a digitally-controlled, ultra-stable work surface–designed specifically for very high-resolution production and metrology applications.

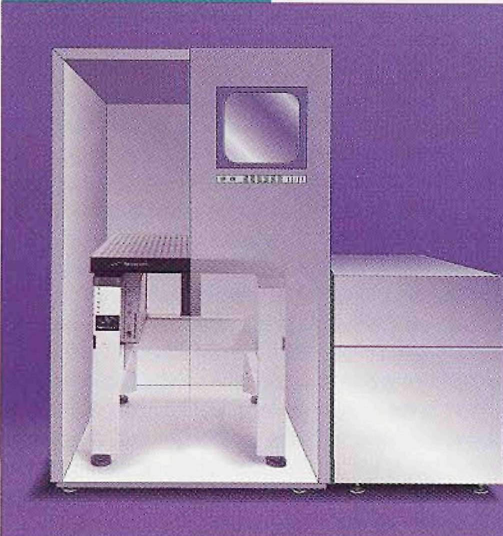
Built on Newport's Elite Series Active Isolation Modules, Elite 3 workstations offer the leading edge in work surface vibration cancellation.

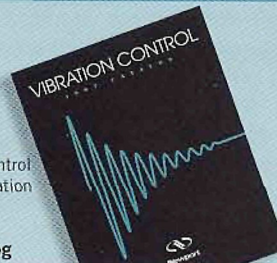
You don't need a crystal ball to see the future of vibration control technology. Call 1-800-222-6440. And let Newport solve your most difficult next generation of vibration problems.

Elite 3 workstations are designed to allow integration into your production and metrology systems.



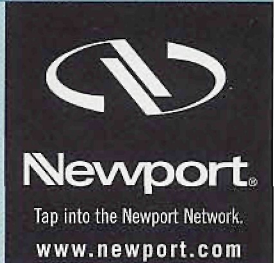
Elite™ Active Isolation Modules are available for integration into OEM production and test systems.





For details on Newport's complete line of vibration control equipment, request our Vibration Control Catalog today.

www.newport.com/catalog



Circle Reader Inquiry #9

USA: 800-222-6440, 714-863-3144, Belgium: 016-402927, Canada: 905-567-0390, France: 1-60916868, Germany: 06151-3621-0, Italy: 02-924-5518, Netherlands: 030-6592111, Switzerland: 41-1-744-50-70, Taiwan R.O.C.: 886-2-506-2366, UK: 01635-521757.

https://doi.org/10.1017/S155192950000565X Published online by Cambridge University Press

A Quick, Economical and Gentle Method for Applying Small Volumes of Solutions to Specimens on Glass Slides

Clare Hasenkampf
University of Toronto at Scarborough

I use immunocytochemical detection schemes that employ the use of primary and secondary antibodies for either tissue sections or whole mount preparations in order to study the *in situ* distribution of proteins and labeled nucleic acids. As the antibodies are either laboriously generated, or represent costly purchases, I have sought a protocol that minimizes the volume of antibody solution needed. Additionally I need a protocol that involves a quick, thorough, and gentle application and removal of the antibody solutions from the specimen with no danger of the specimen drying out during the one or two hour incubations. After trying many various schemes I have settled into the use of a procedure which very effectively retains the antibody solution on the specimen without damaging the delicate sections or whole mounts, and which is easy to add and remove.

My starting material typically is paraffin embedding 5-10 μm sections of lily anthers. I use three sections per slide, and the sections are placed slightly off center. Alternatively I also use whole mount chromosome preparations which are located in the center of a microscope slide within a diameter of 1.5 cm.

I perform the blocking step and antibody reactions by placing the sections (or whole mounts) upside down on top of the solution on a hydrophobic surface. I keep the sections from actually touching the hydrophobic surface by having them suspended across a platform made of two other microscope slides. I have found this technique to be a very reliable, quick and inexpensive way to process my slides gently for immunocytochemistry. I do the antibody incubations for 2 hours and have not had any problems with the solutions drying up or spreading out too far.

The method of setting up the hydrophobic platform area is as follows.

Prepare a hydrophobic work area as follows:

- Pull off a large piece of heavy duty plastic wrap (for example microwavable HandiWrap). Be careful to touch only the edges of the wrap.
- Starting at one end, begin to tape the plastic wrap to a level lab bench surface.
- Proceed to the opposite end, pulling the wrap very taut as you go. Remember touch only the edges of the plastic wrap.

When you have finished you should have a taut, smooth surface with no wrinkles.

Once you have the hydrophobic surface, it's time to create the platforms:

For this you'll need ordinary clean glass microscope slides.

- Take the first microscope slide and place it in the top left corner of your plastic wrap work surface, leaving at least a 1.5 inch border from both top and side edge.
- Now place another microscope slide parallel to it with a distance between the two slides of approximately 1.5 inch.

To apply the solution to the experimental slides:

- Using a micropipette place the desired solution (I use 100-200 μls) on the plastic wrap in the upper, middle region between the two slides of the platform.
- Once the liquid is in place, carefully lower the slide with sections onto the drop, tissue side down.

This should place the sections in contact with the liquid and spread the liquid out somewhat, but the sections will not actually contact the surface of the plastic wrap.

Each such set of two platform slides can be used as the platform to process two slides for immunocytochemistry for one treatment, Figure 1. Thus if you have 10 slides in your experiment you'll need 5 platforms per solution you wish to apply.

To remove the solution from the experimental slide:

- Holding the slide of the relevant portion of the platform, simply lift the experimental slide from the drop, shake it once briskly (there is very little liquid on the slide) and quickly move the slide to its next solution.

I do both my blocking step and my antibody incubations on these platforms. I use 200 μls of blocking reagent at the blocking step, and 100 μls of the antibody mixtures. In this way the region of the slide covered with blocking solution is larger than the region covered by the antibody solution and this helps ensure proper blocking of the slide.

Avoiding problems

1. The spacing of the two platform slides is important. The specific distance to place them apart will depend on where the sections are on the slide, how much of the slide's area the sections occupy, and how much solution you want to use. You can use trial and error with a mock hybridization solution to optimize the spacing for each type of sample.
2. Do not use the same region of the plastic wrap twice. Use a fresh region for each new incubation.
3. Try to get the plastic wrap exactly like you want it before you add any liquid. Trying to pull the wrap tighter after the liquid as been applied can cause dramatic changes in the liquid's location.
4. Before placing the sections on the slide platform, most of any previous solution must have been removed. If the slide is too wet, the antibody solution may spread out too much as it merges with residual liquid. ■

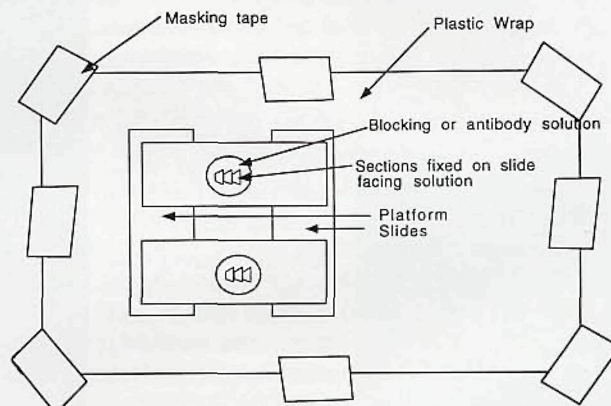


Figure 1: Platform above hydrophobic surface for small volume incubations.

PROTOCOL OF PLANT SPECIMEN PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY

Continued from Page 18

10. Infiltrate in stoppered vial(s) with equal parts of propylene oxide and resin mixture 6-8 hours (or overnight) on rotator. Store the samples in a dry location, but not in a completely sealed chamber where propylene oxide fumes may accumulate dangerously.
11. Infiltrate in **unstoppered** vial(s) with one part propylene oxide, and three parts resin mixture. Continue running vial(s) on rotator for 8-12 hours. Store the samples in a dry location, but not in a completely sealed chamber where propylene oxide fumes may accumulate dangerously.
12. Transfer to pure resin mixture for 1-4 hours while rotating.
13. Embed specimens in BEEM™ capsules or in flat embedding molds. BEEM also makes a hard flat mold that fits into a plastic case. Remove the case before putting specimens in the oven. These molds are excellent and can be used over a number of times. If using BEEM capsules, close the lids. If using flat molds, place each one inside of a covered petri dish. Place in 70°C oven for 36 hours. At the end of that time, remove the specimens and allow them to cool for *ca.* 15 minutes prior to checking for correct hardness with razor blade. If too soft, return to the oven for an additional 12-24 hours.
14. Trim and section with the sharpest tools available. Avoid stain precipitates in post-staining techniques. Usually, 10 minutes of 5% aqueous (or methanolic) uranyl acetate, followed by washing and 5-10 minutes of Reynold's lead citrate staining is sufficient. ■

https://doi.org/10.1017/S155192950005656X Published online by Cambridge University Press