

Updated protocol for methacrylate sections
Citations at END of this protocol

as of 3/96

Fixation:

Buffer = 4% paraformaldehyde, 50mM Pipes, 0.5 mM CaCl₂
Fix for 2h at room temp.

Note: we grow plants on nutrient-agar and pour fixative directly on the plate. This keeps the plants submerged. The above fixative works well for microtubules and some other antigens, but may need to be optimized for different tissues/antigens.

Rinse in the same buffer without fixative, 3 x 10'

Harvest roots:

We get best results with a Formvar loop method. In this method, a loop of copper wire (36 gauge) is made and flattened between two flat pieces of steel. Then small rectangles of 0.25% Formvar in ethylene chloride are floated on water and the loop plunged into the middle of the rectangle so that a film of Formvar surrounds the wire loop. A number of loops are made in advance. A root is then placed on the Formvar surface, the excess cut away with a razor and then this assembly is coated with another Formvar layer, in the same was as above, thus encasing the root between Formvar. This procedure provides better flat embedding than agarose. Up to three loops can be put in a single vial. Note: one does not need super "EM" grade Formvar films, so this is not a hassle at all.

Alternatively, one can use agarose. Excise a 3mm tip segment and encase it within a small droplet of 2% low gelling-temperature agarose (Type VII from Sigma). When the agarose sets, the root-containing drop is transferred to 10% ethanol. The purpose of this agarose encasement is to facilitate exchange of solutions without loss of samples. When all samples have been placed in 10 % ethanol, remove 10% etoh, add 25% etoh.

Dehydration:

25% etoh	30 min	4 degrees C
50% "	"	-20 "
75% "	"	"
95% "	"	"
100% "	"	"

Note: the Planta paper calls for the etoh series to contain DTT. We have found this to be un-necessary and perhaps deleterious.

Add a small volume of a saturated ethanolic Fast Green solution to the samples when in 100% ethanol, as described in the Planta paper.

Infiltration:

25%	methacrylate: ethanol	30 min	-20 deg C
50%	" "	"	"
75%	" "	"	"
100%	methacrylate	overnight	"
100%	"	1 hr	"
100%	"	2hr	"

Note: the methacrylate mix is made exactly as described in the Planta paper. It contains 80% butylmethacrylate, 20% methylmethacrylate, 0.5% benzoin ethyl ether (Aldrich), and 10 mM DTT. Be sure to de-gas mixture with Nitrogen gas for 20 - 30 minutes (preferably, before adding catalyst).

Embedding:

We use beem capsules which we have removed the conical bottom and replaced with an additional cap. This bottom cap is sealed with a small strip of parafilm prior to use. Samples are placed in the capsules so that the sample is flat against the bottom, and the capsule is filled with fresh methacrylate mix and the top snapped on. Taab in England and now Pella in the USA(133-1) sells capsules with flat bottoms that work great. Care is needed to keep the inevitable air bubble as small as possible. Samples are placed under long wave UV (365 nm is optimal). We use a 15W bulb and our capsules are about 5 - 10 cm distant. We have a box for this which we place in the cold room (4 deg C). Polymerization requires 4h, and afterwards, samples are left in the hood overnight.

Sectioning:

The resin can be trimmed and sectioned using standard techniques. We find that the blocks are brittle and that in the early stages of trimming, ie making something that can go into a microtome chuck, care must be taken to avoid fracturing the block. We cut 1.5 - 2 micron thick sections, on a glass knife, dry. You can cut wet sections without any trouble if you like. Sections are transferred to small drops of water on slides, exposed to 60 deg C for a minute to help spread the sections. The slides have previously been coated with 3 - aminopropyltriethoxysilane (Sigma catalog # A-3648. Make a 2% solution of the above in acetone; dip slides in for 1 min; dip slides in 100% acetone for 1 min; dip slides in H2O for 1 min; dip slides again in H2O for 1 min; let air dry. Slides are sticky indefinitely.).

Staining:

Sections are extracted in fresh acetone for 10 minutes (do not reuse acetone for this purpose), and then placed directly in PBS, and rinsed 3 x 1 min. Sections are rinsed for 15 min in 0.1 % Tween 20 in PBS. Primary antibody is applied and incubated at 37 deg for 2h. Sections are rinsed in PBS with 0.05 % tween 20 3 x 10 min. Secondary antibody is applied and incubated at 37 deg for 2 h. Sections are rinsed as for

primary. If desired, a counterstain for DNA or cell walls can be applied to the penultimate rinse. Sections are then mounted. Note, we dilute antibodies in: PBS plus 1% BSA, 0.1% Azide and 0.05 % Tween 20.

Note: we have had very good luck using CY-3 conjugated secondary. These are available from Jackson ImmunoResearch. Fluorescence from CY-3 is readily observed with standard rhodamine filter sets. A good mounting medium for CY-3 is called Vecta shield, available from Vector labs. For localizing microtubules, we have found that Fab antibody fragments are superior to whole antibodies.

Citations

Baskin, TI; Busby, CH; Fowke, LC; Sammut, M; Gubler, F (1992) Improvements in immunostaining samples embedded in methacrylate: Localization of microtubules and other antigens throughout developing organs in plants of diverse taxa. **Planta**, 187: 405 - 413.

Baskin, TI; Wilson, JE; Cork, A; Williamson, RE (1994) Morphology and microtubule organization in *Arabidopsis* roots exposed to oryzalin or taxol. **Plant Cell Physiol.** 35: 935 - 944.

Baskin, TI; Wilson JE (1997) Inhibitors of protein kinases and phosphatases alter root morphology and disorganize cortical microtubules. **Plant Physiology**, 113: 493 - 502.

For in situs, see: Kronenburger et al. 1993 **Cell Biol Internat.** 17: 1013 - 1021.

For the extention of this system for cryofixation, see

Baskin, TI; Miller, DD; Vos, JW; Wilson, JE; Hepler, PK (1996) Cryofixing single cells and multicellular specimens enhances structure & immunocytochemistry for light microscopy. **J. Microsc.**, 182: 149 - 161.

If you have good results with this method, I would appreciate hearing about them. My email is: **baskint@missouri.edu** (Tobias Baskin) and fax is: **573 - 882 - 0123**. Also, if you publish a paper with this method, I hope you will cite the Planta or the Plant Physiology paper above.