Fixation and Embedding of Root Segments for Nematode Studies.

Protocol developed by Ann Ellis, compiled by Stan Vitha.

Specimens:

Segments of soil-grown, washed young roots, 3-6mm long, ~1 mm in diameter, with parasitic nematodes at different stages of development.

Precautions:

All handling of chemicals and solutions should be performed in the chemical hood, wearing gloves and a lab coat. The used chemicals are toxic, teratogens or irritants. Used fixative containing acrolein must be neutralized with potassium disulfate before it can be disposed of. Caution must be taken with DMSO-containing solutions. DMSO facilitates rapid penetration (of the fixative) into tissues (including your own body's) even at low concentrations, and can penetrate some protective gloves. Watch for contamination on your skin or gloves; wash contaminated skin with soapy water, replace gloves.

Chemicals

from Electron Microscopy Sciences, unless noted otherwise.

Osmium tetroxide, 4% aqueous solution,
(EMS cat # 19150)

Formaldehyde (paraformaldehyde) 16%, EM grade, sealed in glass ampoules (EMS cat #15710)

Glutaraldehyde, EM grade, 50%, sealed in glass ampoules
(EMS cat # 16320)

Acrolein, EM grade
(EMS cat #10100)

Cacodylate buffer stock, 0.2M, pH 7.4

DMSO (Dimethyl Sulfoxide)
(e.g., EMS cat # 13390)

Propylene oxide (EMS cat # 20400)

CAUTION: Extremely flammable. Carcinogen. Incompatible with acids, bases, oxidizing agents, copper, copper alloys, brass, bronze, iron, metal chlorides, peroxides and a wide variety of other materials. Reacts with compounds containing labile hydrogen.

Fixative: (always prepare fresh before use)

Final concentration	amounts used
0.133 M cacodylate buffer, pH 7.4	6.65 ml 0.2M stock
2% Glutaraldehyde	0.4 ml 50% glutaraldehyde
2% formaldehyde	1.25 ml 16% paraformald.
2% acrolein	0.2 ml
1.5% DMSO	0.15 ml

add ultrafiltered or distilled water to 10 ml

Transfer leftover Glutaraldehyde and Formaldehyde to glass scintillation vials and blow a stream of nitrogen gas over the surface for ~ 1 min to replace oxygen. Seal with Parafilm and store at -20°C.

Instrumentation

Pelco Biowave, a scientific-grade microwave oven with variable power, temperature control and vacuum.

Protocol

1) Osmium vapor prefixation

Using a cut-off plastic transfer pipette, transfer the specimens into a shallow plastic cup, with just enough liquid to keep them wet, and place the cup in a glass Petri dish. Osmium tetroxide solution in another plastic cup is then placed in the same dish, the dish is covered and a beaker with hot water is placed on top to warm it up. Incubate for 15 min.

2) Aldehyde fixation

Transfer specimens to glass scintillation vials and add the freshly prepared fixative. Incubate for 30 minutes, and then place the vials without lids in a vacuum chamber in the Pelco microwave oven. Use program #3:
2 min at 250W power, 2 min off, 2 min at 250W power.
Temperature is set to 20°C, vacuum is set to a 30s on/off cycle.

3) Washing in 0.133 M cacodylate buffer

For 20 ml working buffer, use 13.3 ml 0.2M stock and 6.7 ml water.
Using a Pasteur pipette, remove the fixative solution (take care not to aspirate the specimens) and transfer it to a beaker with some potassium disulfate to neutralize the acrolein. Add ~5 ml buffer to the samples, microwave with vacuum cycle for 1 min (250W power, T = 20°C, vacuum 30s on/off cycle). Replace fresh buffer and repeat the microwave treatment three times (for a total of 4 washes).

4) Storage

Store the fixed samples in a fresh change of buffer at 4°C until all different samples have been collected and fixed (up to several weeks). Proceed then to osmium post-fixation. Note: storage is possible because of acrolein fixation.

5) Post-fixation with Osmium tetroxide

Aspirate the buffer and add 1 ml of 1% osmium tetroxide (just enough to cover the specimens). Microwave for 1 min with the same settings as for washing. Close the vials, seal with Parafilm and store at 4 °C overnight.

6) Washing

Remove osmium tetroxide (dispose into the proper waste container) and add ~5 ml water. Let sit while preparing the first Methanol/water dehydration step.

7) Dehydration

It is essential that the dehydration is done very gradually, otherwise the tissue would shrink and the morphology would be disturbed. Methanol is a more efficient dehydration agent than ethanol. Use methanol/water graded series at 5 % Methanol v/v increments (from 5 to 95 %). Use at least 5 ml per vial. For each step, remove the existing liquid from the vial, add the methanol/water solution. Make sure the specimens do not dry out during the liquid removal. Microwave for 1 min (same settings as for washing). Proceed then to the next dehydration step. After the 95% Methanol step, perform three steps in 100% methanol.

8) Transfer to propylene oxide

Because the embedding resin is not miscible with methanol, the methanol must be replaced with an intermediate solvent, propylene oxide. Caution: propylene oxide is extremely flammable. Work in the chemical hood and avoid any open flame. Use **glass, not plastic** Pasteur pipettes, since propylene oxide would react with the plastic. Remove all methanol from the vials, add enough propylene oxide to cover the specimens. Place on a rotator while mixing the resin.

9) Mixing the Embed812/Araldite502 resin mixture

Always prepare the resin mixture fresh, use the same day. The mixture consists of DDSE (Dodecenyl Succinic Anhydride) and the epoxides, Embed812 and Araldite502. Since W.P.E. (Weight per Epoxide Equivalent) values of the epoxides vary from lot to lot, the only reliable way to ensure reproducible hardness of the blocks is by using the W.P.E. supplied with each bottle. The Anhydride to Epoxy ratio in our resin mix is 0.7:1.

Table 1: Calculation of the resin stock mixture with the current lot of epoxy resins. Note that the WPE number is lot-specific.

	DDSA	Embed812	Araldite502
MW or WPE	MW: 266	WPE: 146	WPE: 232
Anhydride:Epoxy Ratio	0.7	0.5	0.5
Calculation:	266 x 0.7 = 186.2 g	146 x 0.5 = 73 g	232 x 0.5 = 116 g
Total	186.2 + 73 + 116 = 375.2 g		
Percentage of total	186.2/375.2 = 49.6%	73/375.2 = 19.5%	116/375.2 = 30.9%
To make 10 g , use	4.96 g	1.95 g	3.09 g
20 g	9.92 g	3.90 g	6.18 g
30 g	14.88 g	5.85 g	9.27 g
40 g	19.84 g	7.80 g	12.36 g
50 g	24.80 g	9.75 g	15.45 g

1) Weigh Embed812 and Araldite502, stir.
2) Add DDSA. Stir until no mixing striation is visible.
3) Add BDMA (Benzyldimethylamine; an accelerator) at the rate of 0.2ml BDMA /10g stock mixture, stir well.

10) Infiltration:

1) Add 1 volume of the resin mixture to the specimen vials, swirl well to mix the resin with propylene oxide. This gives 50% resin concentration. Place on a rotator for 1-2 h (no less than 1 h).
2) Add 1 volume resin, swirl, place on a rotator for 1-2 h. The resin concentration is now ~ 75%.
3) Remove all propylene oxide/resin mixture, replace with pure resin. Leave on a rotator overnight.
4) Transfer samples to new, clean vials, remove all carryover resin and replace with freshly prepared resin. Rotate overnight.
5) Repeat step 4

11) Embedding

Mix a fresh batch of resin. Create labels for each specimen, using either a pencil or a word processor and a **laser printer** (NOT inkjet, ink would dissolve). Put a label to the bottom of each well of the silicone embedding molds, and overlay with resin. Transfer samples into the molds and arrange using a dissecting scope, if necessary, in desired orientation. Fill up with resin to make a slightly convex surface (the resin will shrink ~5%). To contain possible spills, place the embedding mold in a tray or in a glass Petri dish lid and polymerize in an incubator at 55 °C overnight.
The next day take the molds out of the incubator and allow to cool. Pop out the polymerized blocks from the molds and store in a container of choice.

Reference:

Mollenhaurer, H.H. (1964), Stain Technology, 39, 111.