Gelatinous marine animals: 'Simultaneous' fixation protocol. 8/4/2001 agmoss
Modified from the methods of Sidney Tamm, Boston University.
Used on rhizostomes, ctenophores, salps and siphonophores: *Phyllorhiza punctata, Mnemiopsis, Beroë, Eurhamphea, Bolinopsis, Ocyropsis, Lampea*, and siphonophores.

• **THIS IS A GENERAL PROTOCOL. IT MAY NOT WORK FOR YOU!** •

Fixation preparation:

1. 'Dress down' jellyfish so that small, very thin pieces are available for fixation. Thin sheets of tissue, or very small chunks, work best. We used very thin sheets of gonadal tissue that were ~ monocellular layers. ***Big chunks*** of mesogloea are **INCOMPATIBLE** with this technique and will cause excessive 'port wine' precipitate.
2. Maintain animal tissue pieces in clean fresh sea water at ambient temp, salinity until use.

To fix:

Make up simultaneous fixation solutions using stocks. That way you can vary the salt concentration if you need to.

Stock needed	*Volume added to final mix (mL)*	*Final concentration*
Part A:		
25% glutaraldehyde	2	2.5%
4% paraformaldehyde (Fresh, see below§)	5	1%
1.5 M NaCl*	1 - 2	0.075 - 0.15 M
0.8 M NaCacodylate (pH 7.5)	2	0.2 M**
0.1 M $CaCl_2$	2	0.01 M
Part B:		
4% OsO_4	5 mL	1 %

* from 32 ppt to ~40 ppt ambient sea water. Adjust with water as needed.

** This is high (and expensive). We usually replace with 50 mM. But if fixation is of an unusual organism I keep it high to ensure pH control.

§ 1 g paraformaldehyde heated in diH_2O to 60 ^{o}C for 1-5 min, add 3 drops 1 N NaOH. Chill on ice prior to use. Very smelly and dangerous! Perform only in hood. You can buy very good paraformaldehyde pre-made from any electron microscopy suppliers. It's best to use that if you are either going to a country where you can't use as many precautions as you normally might, or if you are going to sea. It's much less trouble.

Osmium tetroxide (OsO_4) is very poisonous and can blind you with brief exposure! (It blackens the cornea; wears off after a couple of weeks but is terrifying experience, so I'm told). It blackens everything. Handle with care and especially so if you make the solutions from crystal (which is by far the cheapest way to make it up). OsO_4 solutions last indefinitely (no danger of anything contaminating them!) And can be safely stored in the fridge in a TIGHTLY stoppered brown glass jar. Foil will do too, keep the volume as small as possible in case of a spill. If spilled neutralize with vegetable oil - when it's all turned black the Os is complexed into the oil and is safe. Discard as indicated by your Safety and Environmental Health Officer.

Paraformaldehyde is a carcinogen. Handle accordingly. Keep hot solution in hood.

Fixation protocol

1. **Mix** A and B at the rate of 15 mL of A to 5 mL of B. Do so ONLY ON ICE (See above why)
2. **Fix:** Immediately add to sample, and KEEP ON ICE for ½ hour to 1 hour. Go as long as possible or until you see beginning of 'port wine' precipitate (it's very obvious and is distinctly the color of port wine)
3. **Buffer Wash:** Immediately upon seeing any port wine color, or at the end of 1 hour, wash in ICE COLD Buffer Wash (it MUST be kept ON ICE):

Stock needed	Volume added to final mix (mL)	Final concentration
0.4 M NaCacodylate (pH 7.5)	10 mL	0.2 M
0.6 M NaCl	10 mL	0.3 M
	20 mL	

4. **Osmium postfix.**

4% OsO_4	5 mL	1%
0.4 M NaCacodylate (pH 7.5)	5 mL	0.1 M
0.075 M NaCl	10 mL	0.38 M

Fix in postfix ON ICE for 15-30 min or until the preparation is distinctly blacker. Stop if there is any sign of port wine precipitate.

5. **Deionized water washes**

Keep preparation on ice, 5 rinses of 5-10 min each.

6. **Overnight en bloc uranyl acetate staining.**

This technique can be omitted if it proves to be problematic. Sometimes excess mesogloea can cause precipitation of UA and OsO_4 complexes, very nasty.

Precipitates are quite possible with this technique. This happens if 1) the temperature rises above ice cold temps, or 2) if there is a lot of mesogloea.

Precipitates are bad for EM: cause artifactual bits in TEM and amorphous junk on SEMs.

Once you have reached this point you can now go to TEM or SEM, as you like.

7. **Whether TEM or SEM you must dehydrate: Use EtOH to begin:**

30%, 50%, 70%, 90%, 90%, 95%, 95%, 95%, 100%, 100%, 100%

then 100% times three (as above) with microporous pellets - i.e. really anhydrous!

FINAL PREPARATION for VIEWING:

TEM:

8. PO: You can then put samples in Propylene Oxide for 3 changes X 10 min. It's optional. If you don't have any then it'll be OK. But it's better if you do.

9. Embed in flat aluminum weigh pans: The pan allows you to arrange the tissue, which could be quite broad so long as it's thin. Spurr's Resin works well. Use as per the recommended instructions....even so, because this is for jellyfish, which are so delicate, still 'cut' the Spurr's resin with either EtOH or PO 1:1 initially. Let it sit in that solution for a while, remove and put in 100% Spurrs for several hours. Change out of that (pull the sample out with toothpicks). You can alternatively use EPON or EPON-Araldite. Or, as I use, FLUKA Durcupan. Again, cut 1:1 with the solvent (with Durcupan it's best to put the sample in either PO (better) or acetone (OK) and dry it down under a gooseneck lamp overnight in a hood (all epoxy resins are carcinogenic and so do this in a hood and be sure to wear gloves; treat with great care).

10. Cook at the recommended temperature. 55+ ^{O}C for Spurrs or 65+ ^{O}C for Durcupan. Leave for 3-5 days. Test for 'stickiness' with a toothpick. You can pull the sample test it after it's cooled (it hardens a lot when it cools) and put back in to continue to harden if it's not hard enough. Plastic should be about as hard as a polypropylene test tube. Slightly deformable with your thumbnail but not really hard (like styrene plastics are). The cooking conditions must be very dry or else the plastic will never get very hard.

SEM:

8. Exchange specimen into Hexamethyldisilazane and dehydrate to completion preparatory to sputter coating: HMDS, cat 16,700 from Electron Microscopy Sciences. Exchange twice more after 10 minutes each. The volumes may be very small. Then, leave just enough in the vial to cover the sample, put in the hood, and tilt to allow the upper surface (the one you want to sputter coat to observe) to be up. Then leave it overnight in the hood so that the opening of the vial is away from the entry of air and faces toward the back of the hood (so no bits of junk land on it). Wait a day or so 'til dry. It'll shrink like hell. You'll think it's disappear. Remember, jellyfish are ~96-99% water and the HMDS is the last 'watery' thing the tissue sees, so when it dries the tissue will shrink badly. But you can still get some very cool and very valid images of relatively small and stiff things, like nematocysts etc.

You can critical point dry the sample but I've found that the CP dryers brutalize the delicate jellyfish tissue. Up to you...

9. Sputter coat. This is so specific to the lab that I can't give guidance here.