Biological TEM Introduction and Sample Protocol

Introduction

Preparing biological samples for light or electron microscopy can be very challenging. The goal is to view what we hope represents the living state. You are taking a delicate living cell/tissue and killing it; you hope your process will stabilize and render for observation it in a relatively life-like state. But all processes (other than light microscopy of living cells) that are used for viewing biological structure will in some degree compromise the structure of the living state. With conventional chemical preparation methods you are disabling ion pumping, cross-linking and/or precipitating proteins, chemically stabilizing lipids; there can be swelling, collapse, extraction, rearrangements. Cryo-fixation techniques can avoid some of the chemical fixation issues but can have artifacts as well and cryo-fixation methods are only possible with very small tissues or single cells. For TEM observation we can typically only look at thin sections (50nm-90nm) to allow penetration of the electrons. If sections are to be produced, the fixed tissue is further subjected to harsh solvents, epoxies or acrylics (for embedded tissue). When it all works well you can have a result that looks much like what we think living cells should look like, but there are essentially always some artifacts of the preparation this can lead to a misinterpretation of the material you are studying. It is very important to know what well-preserved cells look like, and evaluate your results critically for significant artifacts; if the problems are significant you must try again, changing some parameters to try to produce a more acceptable result.There are so many fixatives, buffers, osmotic stabilizers, temperatures, pH choices, additives, dehydration solvents, embedding resins and methods that the job can seem daunting. You may get very lucky on the first try, but often it takes some repeated attempts to get things right. Just remember, experience is want you get when you didn't get what you wanted. I know that 5 people executing the same protocol will end up with tissue with 5 different "looks"; there are very subtle things in how a person handles a sample that make a lot of difference. I can absolutely tell you that the microscopy work you do after one year of many hours of consistent work will look very different and be of a higher quality than the work you do your first and second try and the work you would do in 10 years on will

show similar improvement. It doesn't matter how expertly you are instructed, it simply takes time for you to aquire the skills and consistency in your work that produces high quality results. So if you want to do electron microscopy, relax, be calm, pay attention to details and stick with it.
Note: Most of these same issues apply to preparation of biological tissues for SEM.

So how do you start? My advice is to start with some tissue that is not too difficult so you can get through the procedure with something useful to demonstrate this beginning level of mastery. I often recommend plant root tips (I use tips from alfalfa sprouts) since they are easily available, don't have significant issues for penetration of fixatives and buffers (unlike leaves with waxy cuticles, etc.) and the cells are not extremely delicate, and the cells are fairly interesting to examine when you get to the TEM. If you are lucky enough to have access to cultured mammalian cells, these can also be easy to work with either on coverglasses or as a pellet of harvested cells. Some materials are extremely delicate or extremely tough and you should avoid these in the beginning (or middle, or end of your career if at all possible.....). Starting with something simple and basic will probably give you some rewards for your efforts and that is a good first step.

But, because of time constraints or other pressures, many of you are not going to follow this advice and will jump right in with a more difficult project of some sort. For you, I have the following advice. Start simple and build. Don't do an insanely complex experiment with many samples and lots of time points and different treatment variations as your first effort. Spend the time to study some published methods used on your target organism. Use a protocol from a recent paper on the same organism where the images look very good; there is a lot of bad work out there, choose a good work to base your attempt on. Read through several papers and compare the methods and make sure you understand why you are doing each step. Think about timing - where you can break the process to leave it - typically needed except for some very few procedures that can be done in one day. A good book can be an invaluable reference; while there are many books available, **Electron Microscopy - Principles and Techniques for Biologists by John Bozzola and Lonnie Russell (Jones and Bartlett)** is an excellent reference for basic

biological preps and TEM and SEM instrumentation. This is available as an ebook from the Umass library. There are "typical" preparation protocols for plant and animal tissue, for TEM and SEM.

There are very many preparation methods including chemical fixation, cryofixation (where the sample is first immobilized by very rapid freezing) and many variations combining these. There are some methods for electron microscopic (TEM and SEM) observation of living organisms in aqueous medium by the use of special chambers. The cryofixation methods are usually restricted to very small samples and the conditions for the freezing (small samples <10um thick, or something up to a few hundred micrometers for hyperbaric freezing) restricts the use of cryofixation. The goal to study samples in a life-like state is almost always a goal that is only approached. But anything that might be said here about limitations is an avenue of research to overcome those limitations, so current publications, trade journals, vendor shows and the ever-cursed email spam by vendors will keep you informed about the current methods; just be aware that many "latest and greatest" methods that come out have severe limitations for general applicability and often solve one tiny problem well while the old standard methods still provide the main avenue for most sample preparation.

For the lab you will see samples in various stages of a "typical, normal" preparation for TEM sectioning, and then you will see some TEM images of the tissue.

Preparing Plant tissue for thin sectioning and TEM (a typical protocol)

Preliminary notes. Wear proper gloves and work in a good fume hood when appropriate! Glutaraldehyde is a a protein cross-linking agent and should be handled to minimize breathing fumes or skin contact. Osmium tetroxide (OsO_4) is highly volatile and reacts with lipids of mucous membranes (eyes, respiratory) instantly on contact: it MUST be handled only in a good fume hood draft. Many protocols use cacodylate buffer (dimethyl arsenate) and this is toxic and probably carcinogenic and will penetrate the skin. *Minimize volumes used to minimize hazardous wastes generated and dispose of wastes properly.*

Protocol:

1) Samples < 2 x 2 mm, cut with sharp razor blade, put immediately in the fixative.
2) Fixation - 2% glutaraldehyde in 50mM Na-Phosphate buffer (pH=7.2) and 0.5% NaCl - 3h at room. temperature, and leave in refrigerator overnight.
3) washing - 6x with buffer, on rotator, 15-30 min each change. Must remove all glutaraldehyde! Use a rotator to speed equilibration.
4) 1% OsO_4 in 50mM Na-Phosphate buffer (pH 7.0), overnight in fridge (~ 4C)
5) washing - 6x with distilled water. (Proteins are stable now, buffer not needed.)
6) Dehydration in ethanol series - 30,50,70,80,95% - 20min each, then remove 95% ethanol, leaving a small amount, and add equal vol 100% acetone for 15 min. Replace with 100% acetone; repeat 100% Acetone changes 2x more. Acetone is often used instead of propylene oxide and is far less hazardous - definitely worth trying on your tissue. Acetone, or Propylene oxide should be very dry - see notes below about drying over molecular sieve.
7) Prepare the epoxy embedding medium according to instructions provided.. See other notes below.
8) Infiltration - add the Spurr medium gradually; typically 1:3, 1:1, 3:1 ratios (epoxy:solvent) are used for 1-3hr each. Often people add additional more gradual steps, but avoid extending the process excessively. Use rotator.
9) Remove last resin:solvent and replace with fresh resin - 1.5h. Use rotator.
10) Replace resin and leave in overnight on rotator.
11) Embed in fresh medium in embedding molds at 60-65°C overnight. Polyethylene molds are best.

Samples are mounted appropriately for sectioning. Some embedding molds allow direct mounting and trimming. Other samples must be cut out and glued (standard 2-part 5 minute epoxy) to a "stub" with the necessary alignment for sectioning.

Samples are trimmed to a "pyramid" for sectioning. Block faces should be less than 1mm; typically a trapezoidal block face is used. Smaller block faces section more easily - 0.5mm is a good size. The pyramid shape adds stiffness for sectioning; do not make it tall and skinny - use a 45 to 60 degree side angle.Trim down into the sample manually to the depth needed for starting - this saves time.

Knives for sectioning are either diamond or made from broken glass (precisely broken....) and have a trough of water to collect the sections coming off the cutting edge.

Operation of the ultramicrotome instrument for sectioning requires separate instruction, and comfort and skill with operation come from much practice. The general steps are given below.

"Face" the block on a "bad" part of your knife to get a flat face and collect a section for evaluation. Realign and trim deeper as needed. Using a glass knife you may section 0.5um to 2um for facing and “semi-thin” sections. Do not cut sections thicker than about 200nm on a good diamond knife!

Section the block at 60nm (typ) on a good part of the knife, and let a ribbon of sections form in the trough. Sections are expanded to remove compression by waving a stick soaked with chloroform just over the ribbon of sections.

Sections can be picked up on a 200mesh (or finer) grid with no additional section support. Grids with larger openings typically require a support film (typically Formvar, or Butvar, or Polystyrene, or Collodion, or carbon). Films scatter electrons and reduce contrast so use them only when required.

Sections are typically stained with heavy atoms to provide electron scattering for contrast in the electron microscope: 15 min in 2% aqueous Uranyl Acetate, rinsed, 5 min in alkaline lead citrate, rinsed, is a common method. The stains are only slightly specific. Always use the UAc stain first. TEM stains are not highly specific.

Notes:

This short procedure above is an overview, not a substitute for reading a good text on the process that covers all the general principles, material hazards and safety concerns; many of the materials used are very dangerous. Know how you will handle all materials and wastes before starting. If at all possible avoid really problematic reagents like picric acid.

Minimize volumes of toxic materials and handle all toxics properly.

Tissue size is ideally ~ 0.5mm max to allow faster penetration, but some cells can be very elongate and so a larger piece of tissue must be cut so that intact cells are included in the block. You have to obtain a block where the cells are not physically damaged.

Tissue must always remain in liquid, never allowed to be dry, and this is especially critical in the higher concentrations of volatile solvents. Solution changes should ideally be gradual and continuous.

Use solution changes of ~10x - 20x the tissue volume to avoid dilution effects.

Agitation on a "lab rotator" (~2rpm, 45degree inclination) is good for all steps in liquid to enhance mixing.

Final solvent (ethanol, acetone, or propylene oxide) should be DRY. Store over baked-out Type3A molecular sieves that will absorb water. Bake sieves in shallow pan at 260C and cool in glass dessicator. Use of a small heating mantle with a Variac at about 60% (check) will allow 250C to be reached very efficiently. Use ~0.05-0.1 vol in solvent bottle.

Osmium fixation can be much shorter duration depending on tissue size and permeability/density. Osmium reacts almost instantly, but getting the osmium to the inner cells is often an issue since the rate of penetration is slow. The long treatment at lower temperature allows better penetration without badly overfixing the outer cells - it is a compromise for botanical tissue. Cultured mammalian cell monolayers are fully "osmicated" in minutes.

There is no mention here about osmotic stabilizers. Cells have semi-permeable membranes. Cells are subject to shrinkage or swelling if the external medium does not match the internal. The osmotic effect of fixative and buffer components is

often calculated or measured, and the value used can have a significant effect on the outcome. This consideration is often neglected, but it can be very important. Books contain discussions of this aspect.

In working with epoxy resins, the usual reasons for difficulties are allowing moisture to get in the resin (or components) and failure to mix the components adequately. Epoxy resins are typically avaliable as a kit of about 4 components. Measure components fairly accurately by weight, taking care not to drip resin all over the bench and balance. Mix all components well before adding catalyst and mix well again immediately after adding catalyst; you are mixing fairly viscous materials and trying to get a uniform association of the components so mix at least twice as long as it takes to see the component "phases" disappear; you cannot reasonably overdo this step. Keep closed to avoid moisture. Consider mixing smaller batches than the standard formulas supplied - as soon as the catalyst is mixed in that resin has a limited life and becomes more viscous. I mix 10 g batches of "Spurr's" resin in a 50ml dispo centrifuge tube. Any not needed directly can be put in a -20 C freezer (overnight to weeks); let it reach room temperature before opening to avoid condensation and moisture problems. (Sure cooling causes condensation, but the air in the tube has whatever moisture it originally had and cooling doesn't change this. Just make sure the cap is tight, maybe Parafilm seal it if uncertain.)

Spurr's resin formula and general protocol.

ERL 4221 10 g
DER 736 8 g
NSA 25 g
DMAE 0.3 g
Cure Time at 70°C 8 hours
Pot Life 3-4 days

Mixing Instructions:

Add each component in turn to a disposable plastic beaker. An exact weight is recommended, and care must be used in dispensing the final amounts of each component so that no excess is added.

The catalyst (DMAE) should be added last, after gently mixing the three other components.

The complete formula should be mixed thoroughly.

The complete mixture with the hardener can be used immediately for infiltration, and then for embedding. Although the mixture can be stored in a disposable syringe,

well capped and with no air, in a freezer for several months it is highly recommended that freshly prepared embedding medium always be used. If you choose to store the mixture, it is imperative that you warm it thoroughly prior to use.

Dehydration - Infiltration and Polymerization:
This embedding media is compatible with all dehydrating agents: acetone, dioxane, ethanol, hexyleneglycol, isopropyl alcohol, propylene oxide, tert-butyl alcohol. The schedule and concentration can be established by the investigator however for a rule of thumb we recommend the following:
Dehydration is generally done at room temperature in a graded series of Ethanol starting at 50% then going to 70, 95 and 100% for no less than 20-30 minutes each step. All dehydrating agent must totally be removed during infiltration due to the fact that it will effect curing.

The embedding media is completely compatible with ethanol. Thus, it is not mandatory to have a change to propylene oxide prior to infiltration as is true for other epoxy resin mixtures. If working with plant cells it is recommended to use propylene oxide.

The infiltration (one should employ a specimen rotator) can be started by adding the embedding media to the dehydrating fluid left in the vial with the tissue(1:2) . Swirl the mixture and allow it to stand for 2- 3 hours. Then replace with a 1:1 dehydrating agent/embedding medium, swirl and allow to sit overnight. Replace with a 1:3 dehydrating agent/embedding medium, swirl, and allow it to stand for another 2 to 3 hours. Pour and drain the mixture and add fresh embedding media (100% Spurr). For small specimens, 5-6hours; for large specimens, 5-6 hours followed by overnight. Curing takes 16-24 hours at 60°C. (The mixture can be left in an oven overnight).

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Sample protocol for mammalian cells grown on glass slides.

Human Colon Cancer cells, DLD1
(Client did the glutaraldehyde fixation steps, so these details are from an emailed protocol...)
Cells were cultured in multi-well slide-mounted chambers (removable plastic chambers)
Wash cells in cold PBS. (Maybe RT?)
2% glutaraldehyde in 0.1M Phosphate buffer (pH 7.3) 30 min (possibly RT, since the further step is 4C.....)
Further fix at 4C for 30 min
Aspirate the buffer and add 2% glutaraldehyde, 7% sucrose, in 0.1M PIPES for 1 hr at 4C
Aspirate the buffer and add 0.1M PIPES, 7% sucrose
(Shipped to me Overnite (ON) on ice)

Wash with 0.1M PIPES, 7% sucrose; 4C, 30 min
Post-fix 1% Osmium tetroxide in PIPES Sucrose; 4C, 1 hr
Wash with dH20, 3x, 5min ea; 4C/4C/RT
Removed the plastic culture chambers from the slide...
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Dehydrate by 10% increments with acetone
Stored overnight at -20C in 100% acetone.
Brought to RT
Mixed Epon:Araldite resin and diluted some 1:1 in Coplin jar
Transferred one slide of each treatment to the 1:1 mix (others returned to -20C storage (FailSafe)
Agitated gently up and down in jar over 1.5 hr (Infiltration)
Removed slides from Coplin jar (1:1 mix) and placed slide - cells up - on Aclar film and quickly placed 1 drop of 100% resin on each square (culture areas) of slide, and placed in a desiccator chamber to equilibrate.
After 1 hr the desiccator was opened and residual acetone allowed to evaporate for 15 min in fume hood draft.
Desiccator was evacuated, allowed to stand for 2 hr..
Slides were placed in the oven at 60C for the "partial polymerization" method (to enable removal of cells/resin from the glass - separate notes that follow).

Sections were cut (in the plane of the slide) from mounted blocks. ~60nm thickness.

Sections on grids were stained with standard "double stain":
30 min 2% aqueous Uranyl Acetate; rinse;
5 min 2.5 mg/ml alkaline lead citrate (~O.1N NaOH), rinse, dry
Sections are rinsed gently with a flow of ddH2O

The cells were *very* nice.. (dac)

Removing epoxy embedments from glass substrates.

From the Microscopy Listserver.....

Removing embedded cells - partial polymerization method.

We have "popped" cells grown in both serum and serum-free medi on glass coverslips using liquid nitrogen 100's of times. serum makes no difference. The coverslips are fixed either in the 12 well plastic trays or transfered to 20 ml glass scint vials. by the time we get to the dehydration steps, we always switch to the glass vials. after 100% ethanol, we infiltrate with embed 812 (1:1 with ethanol overnight) then 100% resin for 3 hrs then place the coverslip cell side up on a glass slide with the thin layer of resin that comes with it when we transfer to the slide (you don't want it too thin or too thick but it if you are in doubt, simply add 1 drop to the coverslip after transfering and let it flow naturally. you shouldn't get a final plastic spread of more than 1.5 - 2 times the diameter of the coverslip or you are using too much.

When you take the slides out of the oven, let them cool for 15 min and touch with a razor blade. if you get a gooey strand when you pull the blade away, you haven't polymerized it enough. if it is not too gooey, cross-hatch the surface of the plastic using the razor blade to score deeply into the plastic (to the level of the glass). SLOWLY immerse the slide into Liquid N2 and the squares should pop right off. look at the surface of the coverslip and bottom of the square to ensure no glass is going with the square. if so, you have over polymerized. for some projects, we simply put the square in the flat microtome holder and cut it but ususally we re-embed the square in a standard mold. if you aren't re-embedding, you should heat the squares in a rubber mold overnight. once you have the timing of how long to polymerize the coverslips, it is trivial.

we havent had a failure in years of doing this a lot of times. good luck.
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It sounds like you may be already doing this but when we "pop" our cells off ECM coated glass coverslips in liquid nitrogen, we find that it is important to only polymerize for 6 hrs (if we go longer, they don't pop off well)- when we first take the coverslips out of the oven they can be slightly gooey but after they cool for 15-20 min, they are "tacky" but no longer gooey. we cross-hatch the surface with a razor blade and slowly immerse in liquid nitrogen. we have used this for matrigel but not in many years.

Tom Phillips (via Microscopy Listserver)

Dale's notes:

1) **Do** score the plastic! Don't expect to get off whole sheets and cut it later. I got severe crazing of the resin when I didn't score it first. Scoring seems to releive stresses....

2) Don't actually put into liquid N2! Hold it just above the surface of the liquid (down in a cup, etc.) for about a minute. I had better results this way; more repeatable than "slowly immerse in liquid nitrogen....."

3) I polymerized for about 8 hr. and moved to a dessicator until the following day.... This may have been a bit longer than optimal - they were pretty well set; soft, but not tacky..... but it worked fine. The timing of this is going to depend on the temperature, the resin, and the amount of accelerator added.