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Selected postings from the Microscopy Listserver from June 15, 2008 to August 15, 2008. Complete listings and subscription information can be obtained at http://www.microscopy.com. Postings may have been edited to conserve space or for clarity.

SPECIMEN PREPARATION – propylene oxide

The safety folks here are starting to give the evil eye to propylene oxide. In the past, I've skipped the propylene oxide as an intermediate and used ethanol or acetone in the infiltration steps. What is the consensus, or is there one? The less toxic/flammable the better. Paula Sicurello vapatpxs@yahoo.com Wed Jul 2

If you look at nice work that has been published and take the minimalist stance, then you have to accept that acetone is perfectly capable of yielding beautiful results when used as the dehydrating and infiltration medium for epoxy resins. I think I read in Hayat that it is less hygroscopic than ethanol and has some other benefits. There may be some specific cases where propylene oxide or another solvent/combination could be better, but I have seen enough to believe that acetone is probably a perfectly good solvent in almost all cases. Along these same minimalist lines, I have seen enough excellent work using room temperature dehydrations that I wouldn't routinely bother with dehydrations in the cold except for very delicate samples. Here there is a slightly better case for the trouble of cold dehydration, since most materials become stiffer at lower temps and it may protect from some tissue distortions. My advice is probably worth exactly what you paid, Dale Callaham dac@research.umass.edu Wed Jul 2

I don't know the reference, but I recall being told that one advantage of using propylene oxide is that the molecule gets incorporated into the polymerized resin. This is not so for other solvents, which can lead to localized regions of poorly polymerized resin. If you are careful, acetone and ethanol should work fine. John Chandler jpchandl@mines.edu Wed Jul 2

If someone has that reference, I'd be interested. I used to believe that the advantage of propylene oxide is that it evaporates without a trace, which is easy to believe for anyone who has ever handled propylene oxide. We are fortunate here, no one tells us what to use or not use for dehydration and infiltration. Propylene oxide gives you piece of mind, all my infiltration problems disappeared and never came back since I started using it nine years ago. With all due respect, Hayat books (I have almost all) are really a vast collection of recipes and anecdotes, many of which contradict each other. I wouldn't refer to them as a bible. There are much better sources these days, like Bozzola/Russel, Afzelius/Maunsbach... For Epon analogs, my understanding is one should use at least acetone, ethanol alone won't do. I have never tried going from ethanol straight to Epon but have noticed that when the acetone is not freshly opened, I often have infiltration issues. I would use those small bottles of glass-distilled acetone sold by EM vendors and open a fresh bottle every time. A few teaching EM labs I've been to use molecular sieves in bottles of pure ethanol and acetone to keep them dry. Reportedly, this can cause problems with diamond knives. It all depends on what you must infiltrate, of course. While you can do just fine without propylene oxide with mammalian cell culture and many tissues, skin will be more difficult. Vlad Speransky vladislav_speransky@nih.gov Wed Jul 2

The Hayat books do seem to take all possible sides of every issue. Propylene oxide is an epoxide and I've been told that rather than helping if there are traces left, it reacts without crosslinking and so it is likely to be worse if residual propylene oxide is left relative to other solvents. The high evaporation rate of propylene oxide causes cooling and condensation of moisture if used when open in a fume hood draft. Since I work in a facility, I have to repeat methods that people request whether I see the logic to it or not, so I do use all manner of solvents. They can all work well. If you look at some of the classic ultrastructure papers, you will see that people have successfully done just about everything. I always use a Type 3A molecular sieve in the bottom of my bottles of any solvent and reserve these bottles for the final two changes - and have threatened bad things on anyone who shakes the bottles. I initially wash the molecular sieves to remove any fines. I bake each charge of molecular sieve (~5% by vol) at 250°C+ in a fume hood using a hemispherical heating mantle on a Variac set to give the correct temp (depends on the mantle wattage) measured with a thermocouple. The molecular sieve is heated in an aluminum dish with a loose cover in the fume hood. After 2 hours "at temp" the sieves are placed on a porcelain support in a glass desiccator and evacuated for cooling - so it doesn't pick up moisture - and transferred to the bottle (Quorpak, with polyseal closures) as soon as cool. The solvent is added to fill the bottle and it is left at least overnight to settle and equilibrate. I pipet from well above the sieves and discard the solvent dregs, drying and reusing the sieves: http://www.bio.umass.edu/microscopy/mol_sieves.htm. As for damaging diamond knives, I am still using the same diamond knife I received (used) when I started working at our facility in 1994 and it has no new knife marks; I do a modest amount of sectioning, and have molecular sieves in all final solvents. I wish we had some solid data on the actual rate that solvents pick up moisture. I think this could be done with a Karl Fischer coulometer setup, but I don't have access to one. We all know the dogma about moisture in solvents, but it would be nice if someone could do some tests like taking one sample from a bottle of dry solvent and then pouring out some and leaving it half full and open/closed and sampling at intervals to see what happens at some typical relative humidity. Lacking hard evidence, I use the molecular sieves for all final changes of solvents in water-sensitive applications and have no moisture problems. Dale Callaham dac@research.umass.edu Wed Jul 2

Here is my experience: I have been using acetone for the infiltration for a few years, and I never have had any problem with tissues. The only problem I got was when I was using gelatin to pre-embed cell suspension or bacteria. After switching to bacto Agar, this was solved. I use ethanol only for dehydration, unless I have a monolayer of cells that were grown on a plastic dish for which I want to use the flat embedding technique. In that case, I do use ethanol all the way from dehydration to infiltration and it works very well. Here is a reference I found about this problem : Edwards HH, Yeh YY, Tarnowski BI, Schonbaum GR. Acetonitrile as a substitute for ethanol/propylene oxide in tissue processing for transmission electron microscopy: comparison of fine structure

and lipid solubility in mouse liver, kidney, and intestine. Microsc Res Tech. 1992 Mar 1;21(1):39-50. Rachid Sougrat sougratr@mail.nih.gov Wed Jul 2

Some months ago someone (don't remember who) recommended acetonitrile as a substitute for propylene oxide. We've tried it in my lab and it seems to work well. I'm in a process of comparing structures, but so far it looks very promising, and the safety officer is very pleased. Randi Olsen randi.olsen@fagmed.uit.no Thu Jul 3

I don't understand how your safety officer can be pleased to see you working with acetonitrile - "The substance is toxic to blood, kidneys, lungs, liver, mucous membranes, gastrointestinal tract, upper respiratory tract, skin, eyes, central nervous system (CNS). The substance may be toxic to the reproductive system. Repeated or prolonged exposure to the substance can produce target organs damage. Repeated exposure to a highly toxic material may produce general deterioration of health by an accumulation in one or many human organs." (extract of the MSDS for acetonitrile, http://www.sciencelab.com/xMSDS-Acetonitrile-9927335). I have already reported that I tried to use acetonitrile during dehydration and embedding with no satisfying results. One has probably to increase the dehydration times. I didn't try ethanol dehydration and acetonitrile embedding, it may be an option. I second the remarks of Rachid concerning the use of ethanol when flat-embedding in Petri dishes. However, in this case it is very important to increase the incubation with epoxy alone, since traces of ethanol may disturb the polymerization. I remember I used acetone in the past, but I concentrated only on the nuclear morphology. Perhaps it has a better extraction property, which may be appreciated (if you want to contrast a given feature) or not (if you want to keep as many structures as possible). But it works. Stephane Nizets nizets2@yahoo.com Thu Jul 3

Many years ago, I had to embed cultures of retinal pigment epithelium grown in plastic dishes. Propylene oxide was out of the question. So after several changes of absolute ethanol, I mixed absolute ethanol with Epon substitute 2:1 then 1:2 and finally several changes of pure Epon. I also used agitation and a time in a vacuum desiccator to be sure I got all of the ethanol out. Worked like a charm. Also, it is possible to skip absolute ethanol completely and go directly to an Epon substitute mixed with 95% ethanol. Labs doing post-embedding immunostaining do this routinely. Geoff McAuliffe mcauliff@umdnj.edu Thu Jul 3

Actually, there was a paper from T. J. Beveridge's lab, back in late 80s, I think, where they compared acetone to ethanol as a dehydration agent by analyzing the exchanged fluids to find out how much stuff gets washed out. There was less extraction with acetone. Still, I almost never dehydrate in acetone, because ethanol is so much nicer to handle. Vlad Speransky vladislav_speransky@nih.gov Thu Jul 3

SPECIMEN PREPARATION - Epon failing to polymerize

I embedded biopsy samples from three individuals in Spurr's epoxy that polymerizes only to a gummy-bear consistency. It's possible that I did not add the catalyst but I am not sure exactly what went wrong. I've polymerized overnight at 70°C; then overnight at 100°C; then for 60 minutes in the microwave all without additional polymerization. Does anyone have an idea on how these difficult to obtain samples might be saved? Douglas R. Keene drk@shcc.org Fri Jun 13

Several of the "standard" microscopy texts talk about dealing with the inevitable problems like this. If it is truly not polymerized you can just extract with solvent like propylene oxide and the re-embed with some test-polymerized resin. There are some methods for removal of polymerized epoxy as well, but these are primarily used on sections and I don't know how long it would take to remove the resin from whole specimen-sized blocks. While it might seem harsh, I think that the chemistry is fairly well targeted to the epoxide linkages and H. Ris showed some very delicate structure using this method (on sections....). I have had similar problems from time to time and anytime possible I keep some samples at -20°C in 100% acetone and leave some at -20°C in the unpolymerized resin. Additionally, I usually do several resin changes including one overnight, so I put some of my resin into a BEEM cap and test harden the resin overnight so I know if there is a major problem with the resin before polymerizing. "Idaware Method" A solution for the removal of resin from epoxy sections. Tsukasa Idaware, Etsuko Harada, Shinji Yoshino, and Taizo Arai. Stain Technology 65 (205). 1990. Stock solution (aka "Treating solution"): 1.3 g 18-crown-6 ether (Aldrich #16,665-1 or Sigma # C5515); 99 ml DMSO; 1 ml water.Working Solution: (prepare fresh daily) 3 ml 30% methanolic potasium methoxide (turbid white; stir to mix, then draw 3 ml); 100 ml Stock solution (above). Sections are attached to "aminosilane" treated glass coverslips or slides. Mix the "working solution"—it should become clear when mixed. Place the glass with sections into the working solution. Leave for 5 min, swirling once a minute. Withdraw the glass and place section side up into distilled water. Wash gently with several changes of dH2O. Various treatments are now possible: - dehydrate and CPD; sputter coat for SEM. - immunolocalization? Notes: Hans Ris used a Polysciences epoxy removal kit which is essentially the Idaware formula. Ris dehydrates 50%, 70%, 80%, and 95% ethanol, and 2x in dry 100% ethanol. He sputters with ~1 nm of platinum for high resolution FESEM observation (Hitachi S-900 FESEM at 1.5 kV; this is an in-lens system). The paper does not state how the methanolic potassium methoxide is prepared, but it is probably like the sodium ethoxide formulas below. Sodium Ethoxide formulas: Ethanol is dehydrated over 3 A molecular sieves. Ethanolic NaOH, 3%, is prepared by dissolving 3g NaOH in 97g anhydrous ethanol. 10% ethanolic NaOH is prepared similarly. Dale dac@research.umass.edu Sat Jun 14

The most likely causes are old accelerator or incorrectly mixed resin. If you are using the "new" ERL 4221 instead of 4206 the quantities may be wrong in the mixing instructions. A straight 1:1 substitution will not do. See the work of E. Anne Ellis for the correct recipe. For your current samples, dissolve out the "bad" resin and re-embed in fresh and correctly mixed resin. Geoff McAuliffe mcauliff@umdnj.edu Mon Jun 16

The references I think Geoff refers to are from Microscopy Today: July 2006, 14(4) Solutions to the Problem of Substitution of ERL 4221 for Vinyl Cyclohexene Dioxide in Spurr Low Viscosity Embedding Formulations. E. Ann Ellis September 2006, 14(5) A Simplified Method for Formulation of Epoxy Resin Embedding Media. If you don't have these, they can be downloaded as pdf files from the website: http://www.microscopy-today.com/MTSelectTOC.html You can only get entire issues, not separate articles, but that shouldn't take long with an ethernet connection. Philip Oshel oshel1pe@cmich.edu Mon Jun 16

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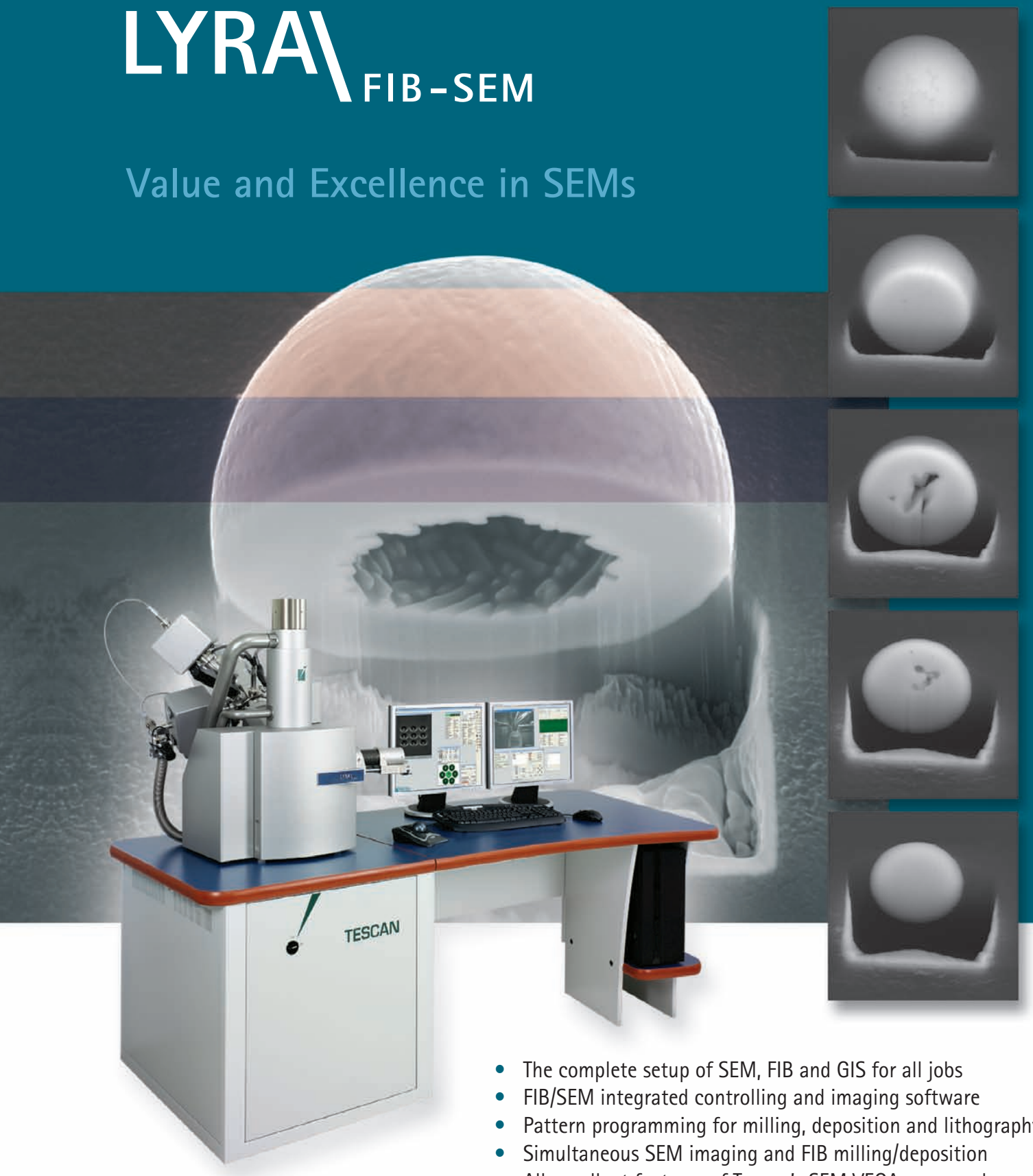

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As someone who has embedded thousands of muscle biopsies, I would suggest that Spurr's is not the best choice. "Epon" type resin mixes are extremely reliable and have excellent staining and cutting characteristics. My standard mixture is Polybed 812: Araldite 502: DDSA in the proportion 5:4:12 by volume (no commercial interest in products). Aliquots can be frozen and used when needed, with DMP added at time of use. I use Spurr's only for special purposes -- plant material, fungi, parasites, etc. Were the bad blocks embedded in flat molds or in BEEM capsules? Spurr's does not polymerize well if the humidity is high. If using flat molds, put them in sealed plastic containers with some Drierite. Ralph Common rcommon@msu.edu Mon Jun 16

SPECIMEN PREPARATION – LR White polymerization

We have a polymerization problem with LR-White in BEEM capsules after polymerization in the heat. In many samples, not in all, and only around the tissue the resin becomes very brittle, the rest of the block is perfect polymerized. What is the reason for this and how is the problem solved? Anne Heller heller@uni-hohenheim.de Mon Jun 23

It could be a couple things, but we have had problems in the past with osmicated tissue causing polymerization problems. I think this is a problem, especially with LR White that has been stored and is near or past its expiration date. In our case, the resin "curdled" and looked very much like clear cottage cheese. We were not able to save the sample. Since the polymerization was fine except for right around the sample, it would appear that oxygen penetrating the BEEM capsule is not the problem. Rather it seems to indicate that something in the sample is causing it. If your sample is not treated with osmium, maybe dehydration was not sufficient, although my understanding is that LRW is tolerant to residual water content. Was there anything unusual in the specimen processing? More detail might be helpful. Randy Tindall tindallr@missouri.edu Mon Jun 23

I would go along with all of Randy's suggestions: re OsO_4, or even if the sample was particularly dark and dense polymerization can be adversely affected. Old reagents: Definitely a possibility, with the catalyst and accelerator prime candidates. Also with regard to the catalyst, some suppliers have altered the stabilizing agent to meet safety regulations for transportation. It may be worthwhile drying the weighed catalyst in a 37°C oven to drive this off before adding to the monomer. It might also be worth your while trying the LR White accelerator method of polymerization - but don't use the suggested proportions in the LR leaflet. With in-date accelerator, (we replace after 6 months), use 1 µl per ml, mix by gentle inversion then completely fill the capsule and fasten the lid. Surround the capsule in a crushed ice heat sink - and don't hang about as the resin will start to set in about 10 minutes and should be complete in 1 - 1.5 hrs (you can check the bubble on top to see if polymerization is complete). Embedding capsules: The harder polycarbonate capsules work best but as the BEEM capsules do not seem to be the problem with your oven cured blocks, they should be OK for the accelerator polymerization method too. Alastair McKinnon a.d.mckinnon@abdn.ac.uk Mon Jun 23

SPECIMEN PREPARATION - tissue culture embedding

We have a customer who has grown cells on Thermonox and needs to see them on edge, so I embedded the Thermonox in flat molds in Eponate 12. However when sectioning, the Thermonox pulled away from the resin. The cells are then just sort of hanging out in space or the edge of the section folds over on itself so the cells are inside the fold. Can anyone recommend a resin or another way to embed the cells so they will stay embedded and not separate when sectioning? I tried picking them up on Formvar but would like to avoid that if possible. Mary Gail Engle From mgengle@email.uky.edu Fri Jul 11

Embed the Thermanox coverslip inverted (cell side down) on a square of Aclar over a drop of Eponate 12. Then I polymerize this as usual. After polymerization I remove the Aclar, it peels away with no effort. Then use a razor blade and remove small rectangles, about 2 mm x 3 mm, from the coverslip, about 3 or 4 pieces. Don't cut completely through the coverslip or it is nearly impossible to remove from the resin. I then use the corner of the razor blade to peel the resin from the coverslip. Pour Eponate 12 into the regular silicone mold and then individually place the cut rectangles in the end of the mold creating a stack. Polymerize the blocks. When you section them, you will get a nice cross section of several cell layers and they will not split apart. Jo Dee Fish jfish@gladstone.ucsf.edu Fri Jul 11

Jo Dee, I read your recent reply to the list with interest, we have had similar problems embedding and sectioning monolayer cells, although we have grown the cells on Aclar instead of Thermanox. I assume the method you described could be employed using 2 pieces of Aclar? Have you ever tried that? David Lowry dlowry@asu.edu Fri July 18

Yes, it works just fine. The only difference is that the polymerized resin will easily peel away from both layers of Aclar, so you can simply cut the resin. Make a thicker drop of resin between the layers of Aclar so it will be easier to work with. Jo Dee Fish jfish@gladstone.ucsf.edu Fri July 18

Since you already have thicker slabs of Epon you can still glue two of them together with some embedding Epon. The best to use is some from the same batch that you used for the original embedding if you have any frozen. Nearly fill an embedding mold with Epon, put in a small rectangle of your embedded cells (of course chose a dense area) with the cells facing up. Let the Epon flow over the top and place a second rectangle of cells, cell side down in a coverslip like manner to avoid air getting trapped between the layers. Fill the mold as usual. Cure for at least 24 hours - two days would be better since the embedding you have is already fully hard. When you go to section make sure that you are into the area of the embedded cells at the bottom of the block face. Sometimes the layers will separate when trimming the bottom especially if the Epon was not the same. You may need to glass knife trim the last bit instead of razor trimming. I had presented a similar work at M&M 1977 but it was for cells embedded in dishes. The take home message was that you can embed both control and experimental cells in the same mold and eliminate differences in section thickness and staining if you trim the block in an identifiable manner. easier to show than to describe!

— Something like this. I put the control on the bottom of the embedding mold and the experimental cells were on top of the

control. Trim the side where the controls are perpendicular to the bottom of the block face instead of making a trapezoid. The block face would be at a strong angle on the other side so that you can readily see on the grid which line of cells is which. I have made block faces over 2 mm long, but not wide, so that only one section was on each grid. Patricia Stranen Connelly connellyps@nhlbi.nih.gov Fri Jul 11

If the samples are fully embedded then the simplest method is to re- embed the blocks in more of the same resin so that the edge is in the middle of a block and just cut as normal. Malcolm Haswell malcolm.haswell@sunderland.ac.uk Mon Jul 14

SPECIMEN PREPARATION – Staining for suberin and lignin

I want to localize and/or differentiate the arrangement of lignin and suberin in plant root cell walls. I have used bright field and fluorescence microscopy to confirm the presence of lignin and suberin. However, I am not sure which technique will be more appropriate to give me more information on the positioning/arrangement of suberin and lignin? Rachel ecoagripolicy@gmail.com Wed Aug 13

The paper by Biggs (1985, Stain Tech. 60: 299-304) differentiates suberin from lignin by assaying for quenching of lignin autofluorescence in suberin via staining with Sudan dyes. This worked for us in study of Casuarina root nodule infected cells, so I can vouch for its efficacy. R. Howard Berg rhberg@danforthcenter.org Wed Aug 13

You can also use berberine sulfate to pick up only suberin - see papers by Mark Brundrett. In cereal roots, crystal violet is a good counterstain that quenches fluorescence from other walls after berberine staining. Rosemary White rosemary.white@csiro.au Wed Aug 13

Biggs used Sudan black B to quench suberin fluorescence and used phoroglucinol HCL to quench lignin autofluorescence enabling good differentiation of the two even when they are localized quite close to each other. We have used this technique extensively in our studies of fruit skins - Another reference is Biggs, 1987, Phytopathology 77:718-725. Ian Hallett ihallett@hortresearch.co.nz Wed Aug 13

SPECIMEN PREPARATION - stain artifact

We are having a tedious stain precipitation problem for one month. We have been using our standard contrasting protocol for years and we never had a contrasting problem before. We used glass knives for sectioning within this time without any serious precipitation problem. Last month, we decided to use our new diamond knife for sectioning and after that, contrasting problems started. It may sound silly, but I really wonder what can cause this problem. When our assistant took gold colored "relatively thick" sections by a handmade glass knife, contrast is ok but resolution is unsatisfactory. When she used the diamond knife and took silver colored ultra thin sections the results are a real disappointment. Precipitates obscuring all structures and preventing even diagnose of patients. Taking photograph for research studies is also impossible. We tried to contrast both kinds of sections simultaneously but nothing changed. We need help, we want to use our diamond knife effectively, because it cost us a fortune. I can send photograph samples with precipitates, if you want to examine. Brief protocol: Float on uranyl acetate drops for 5 min. Wash with plenty of distilled water. Float on Reynold's lead citrate for 2 min. Wash with plenty of distilled water. Dr. Necat Yilmaz nyilmaz@mersin.edu.tr Sat Jun 14

We had a rather similar problem with precipitates and I could not trace it to any conditions for half a year. It was very frustrating! However, the problem was in lead citrate step. Do not float your grids on the droplet but put them inside (let them sink). In this way grids will not have a contact with atmospheric carbon dioxide that can cause rather nasty precipitate. Wash the grids after staining them by holding them with tweezers and soaking into the beaker with distilled water 30-40 times. Then just blot the grid with filter paper. aleksandr.mironov@manchester.ac.uk Sun Jun 15

Reynolds did that to me also at times. Check out Sato's lead stain for a formulation that does not do this. We've used it for over 30 years now. Here, from my and Jan Factor's May 3 2006 posting to the list: Sato's lead stain is a more stable replacement for Reynolds Pb citrate. We've used it since the 1970s. 1968 Sato, T.: J. Electron Microsc. 17:158, 1968. 1968 Sato and others: Proc. XIth Int. Cong. on Electron Microscopy. Kyoto. 1986, pp. 2181-2182. 1968 Takamasa Hanaichi et al. A Stable Lead by Modification of Sato's Method. J. Electron Microsc., Vol. 35. No. 3. 304-306. There were a cluster of postings in May 2006, if you can search under Subject: [Microscopy] TEM--Lead Citrate--HELP!! the most helpful about calcined lead citrate were Jan Factor's second May 3, 2006 posting and Stephane Nizet's of June 7, 2006. Michael K. Reedy mike.reedy@cellbio.duke.edu Mon Jun 16

We also had a mystery precipitate problem in our lab for a long time that defeated all attempts to beat it, until our director suggested adding 2-mercaptoethanol to our processing steps before, during, and after osmium fixation. Problem solved, but cause unknown. I have posted a couple other emails about this to the list. This might not relate to your specific problem, but it's worth a try if you have Intractable Pepper Syndrome and nothing else works. We have a protocol on our website at http://www.emc.missouri.edu/Pdfs/General%202-ME%20Microwave%20Processing %20Protocol.pdf. You can easily adapt this to non-microwave processing. Randy Tindall tindallr@missouri.edu Tue Jun 17

The suggestion of mercaptoethanol is quite interesting. I'm trying hard to think how breaking S-S bonds could impact precipitates. Did you do any controlled comparisons, etc, and could you share the concentrations and way you implemented this into your protocols? Paul R. Hazelton paul_hazelton@umanitoba.ca Tue Jun 17

I second all the advice of Vlad (hope you saw them, Vlad is always very helpful): carbonate-free NaOH, rinsing in diluted NaOH after lead staining. I don't really see how your problem could be dirt if you see it on thin sections and not on thick sections. What you could do is to cut at the same thickness with a glass knife and a diamond knife. For example, both at 200 nm. Then you will at least know if the problem is related to the thickness or to the knife. Stephane Nizets nizets2@yahoo.com Fri Jun 20

I'd like to thank you first, for your kindly efforts to help solving my precipitation problem. I'm very pleased to be a member of the group. I put some sample pictures with precipitation problem for examining on a web page its link given below. I wrote also our detailed solution preparation, processing and contrasting protocols below. Additionally, we checked our sections without contrasting and contrasting with UA only and we didn't find any problem.

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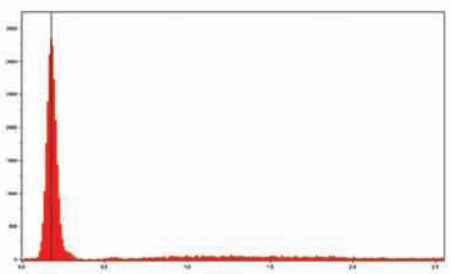

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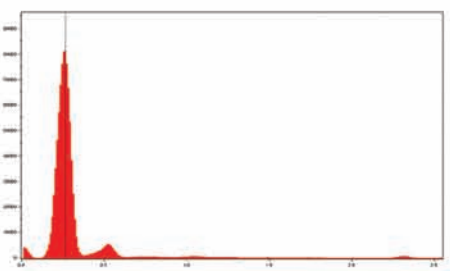

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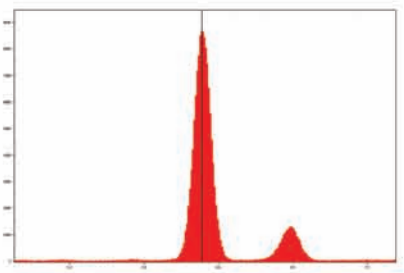

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Thanks in advance. Link: http://electron-microscopy.blogspot.com/ Protocols: Processing: (1) Fixation in 2.5% cold glutaraldehyde, 4-6 hours. (2) Washing in cold phosphate buffer. (3) Post-fixation in 1% cold osmic acid, 1 hour. (4) Washing in cold phosphate buffer. (5) Dehydration in graded cold alcohols. (6) Propylene oxide 2 x 15 min. (7) Propylene oxide + resin mixture 3 x 30 min. (8) Resin with agitation overnight. (9) Embedding Staining: (1) Floating (I don't know why?) on uranyl acetate, 5-10 min. (2) Washing in carbonate-free dH_2O (3) Floating on lead citrate, 2 min. (4) Washing in carbonate-free dH_2O. Preparation of solutions: Uranyl acetate: (1) Saturation in dH_2O. (2) Filtering with Whatman filter paper. (3) Filtering with 0.22 µm membrane filter. Lead Citrate: (1) 1.33 g Lead nitrate. (2) 1.76 g sodium citrate. (3) Mix in 30 ml carbonate-free dH_2O for 30 min. (4) Add 8 ml 1 N NaOH. (5) Add 12 ml carbonate-free dH_2O. Necat Yilmaz nyilmaz@mersin.edu.tr Wed Jun 18

At least one of the images looks similar to a problem that I had (and occasionally still have). Have you looked at an unstained section on the TEM? Can you see spots? I found that some of the spots were there before staining. The problem seems to come from the air. When I work fast to pick up the sections from the boat and limit the air moving around by closing off adjacent doors to the room that my microtome is located in and put a filter over the room air source, I can get large areas that are clean. At times when I get interrupted during my sectioning I can actually see that things have landed on the surface of my knife boat water and the sections that I pick up are dirty. I now start over using fresh water. If I remember correctly, you have recently started using a diamond knife. The boat is so much larger so you are most likely sectioning for a longer time than you had when using glass knives. My water is acidic (not distilled) so I have started to use basic water (1 pellet of NaOH to a pint bottle of water) for the drop that I put the grid into (rather than on) before the lead stain step in my staining protocol. Maybe I imagine that it gives me better results but it seems to be working well. Patricia Stranen Connelly connellyps@nhlbi.nih.gov Wed Jun 18

Patricia Connelly makes some good points: some contamination appears to come from the air. Over the years, I've encountered this problem in labs where rotary pumps were not properly exhausted and microdroplets of oil vapor are present in the air. In one instance, the pump exhaust was attached to a plastic pipe that disappeared into the ceiling, supposedly to be vented outside. Guess what? The pipe terminated in the space above the false ceiling. Likewise, a fume hood that was supposed to exhaust to the outside had a broken vent pipe that was doing the same. Now, such events would result in a major OSHA investigation and lab shutdown, probably. Other sources for air-borne contamination: drive belts from electric motors (notorious for giving off tiny, carbonaceous particles), chemicals on shelves that give off volatiles (ammonium compounds, organic buffers, HCl, for example). Often, the chemical volatiles combine to form fine powders (like NH_4Cl). Store these chemical in a properly vented fume hood. Contamination may also be introduced via the water used for staining and sectioning. Some examples: volatile amines from house distilled water, lubricants used on syringe plungers, humectants used on micropore filters, decomposing micropore filters (due to using old filters or by bacterial degradation of filters that have not been changed), oily contamination in diamond knife boats that have never been cleaned, oily contamination from eyelash probes, improperly cleaned glassware. It's a dirty world out there! John J. Bozzola bozzola@siu.edu Wed Jun 18

Yikes, that's some bad dirt! It does look like something other than the staining protocol, so check and clean your knife (including the boat and the backside of the diamond), your eyelash and forceps and any other tools you use, fill the boat with your best water from a syringe with a new, clean syringe filter, etc. For staining with lead, I always stain within a closed Petri dish with several (or a small pile of) pellets of NaOH on the side to further reduce carbonate. This seems essential in my lab to reduce the really fine pepper. Test your "carbonate-free water" by dipping an unstained grid in it and letting it dry. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Wed Jun 18

Thanks for posting the pictures. This does look like lead precipitation. First, answering your original question - although there is hardly a direct relation to thickness, sections of lesser quality do get dirty much easier. And thinner sections are *definitely* more likely to come out in lower quality. Did you try cutting thicker on the diamond? For all except resolution-demanding work, I personally prefer sections that are just a little bit yellowish, silver is too thin and will be more difficult to stain. I would first experiment switching back and forth, glass to diamond and back, also varying the thickness. Will it still be that thinner sections get dirty? Then it is the lower quality sections "trapping" the lead. There is also a possibility, of course, that your new diamond knife setup got dirty somehow - the boat? edge?.. From your protocol, I see that you do use CO_2-free water already. I will just add that it is also important (from my own tests, long ago) to have your NaOH as CO_2-free as possible. I routinely got Pb contamination from old, long since opened, NaOH granules. Here we now use titrated 1.0 N "CO_2-free" NaOH solution sold by a company, but before that was available, I would keep a special bottle of NaOH granules, for EM lead stain only, and replace it with a freshly opened one regularly. From my experience, this was more important than the extra water you add to make the stain. Dipping instead of floating, mentioned by others, is another measure apparently making a difference. One more thing, you can surround your grids being stained by granules of NaOH - set this arrangement on a lid of the Petri dish and cover with the bottom. Finally, you can try rinsing the grids after staining with water that has some NaOH in it - 0.01-0.02 N. I never had to resort to this, though, and there is, of course, another side to it - your staining may become weaker. Just like if the pH of your Reynolds is too high. Although this is not directly what you are asking, may I suggest a couple more tips to your protocol? They should help with better structural preservation and, likely, better sections: 1) 4-5% glutaraldehyde, instead of 2.5%. (2-4 hours should be enough). 2) During dehydration, include a step of 1.5% uranyl acetate in 70% ethanol, overnight in the refrigerator. Then you can skip uranyl acetate staining of the sections. 3) From the pictures, you seem to have some infiltration issues. Consider modifying your infiltration schedule. A lot will depend on how you actually handle things, fluid changes and all, but you may be going through your propylene oxide/resin mixtures too fast – 3 x30 min? Try leaving the samples in one of the mixtures, 1:1, or, better, 1:3 PO:epoxy overnight, and in other mixtures for ~2 h. Then, finally, 2-4 h (less critical) in pure

resin, then into embedding molds with fresh resin and right into 60°C oven. Catalyzed epoxy thickens quickly, so it is a good idea to make it without the catalyst (BDMA? DMP30?) and add it to one portion before making mixtures for infiltration and to another portion the next day, to have the resin fresh and less viscous. Vlad Speransky vladislav_speransky@nih.gov Wed Jun 18

As I promised I'm sharing our experiences about contrasting problem: We definitely figured out that the problem was due to uranyl acetate. We tried your advice and stained our sections with only uranyl, only lead and also tried without staining. Our results were clear that precipitates are due to the uranyl acetate step. We also tried staining with modifying UA step (more careful manipulations) and results were great. I'd like to thank all of my colleagues who helped with our contrast problem. Necat Yilmaz nyilmaz@mersin.edu.tr Thu Jul 10

SPECIMEN PREPARATION - black precipitate in sperm TEM sections

I have been preparing Drosophila sperm in and out of seminal vesicles and found that a black precipitate is present in the tissue. It is either fine grain particles or very dense particles up to about 60nm in size. Resin only areas of a section are clean. The tissue was prepared using glutaraldehyde in sodium cacodylate, osmium, encapsulation of pellet in agarose, uranyl acetate block stain, acetone dehydration & embedding in Spurr's resin. I used no section staining. The structure of the sperm is great - the precipitate ruins everything! Other tissues I've prepared recently are clear of precipitate. Ursula Potter u.j.potter@bath.ac.uk Wed Jul 9

I suspect that there is incomplete dehydration. I have done similar work on testis embedded in Epon and had exceptional micrographs but used times for dehydration that were a bit longer than for other Drosophila tissues. Is your acetone a dry one (water 0.1%) like Mallinckrodt 2440? From a bottle that has not been open long? Have you looked at sperm as soon as the beam hit them? If so, can you see the precipitation forming as the spot heats up? This happened when I had trouble with bacteria inside cells getting the precipitate just over them and the centrioles. It was solved by increasing the dehydration time slightly and by an additional 100% step from a bottle that had not been opened more than a few times. You do not mention that you used tannic acid so there should be no problem there. Precipitation does not bring back good memories! Patricia Stranen Connelly connellyps@nhlbi.nih.gov Wed Jul 9

You didn't mention osmium fixation! Uranyl can precipitate, but the precipitate doesn't match with your description of "very dense particles up to about 60 nm. I would advise (1) to try without uranyl en bloc staining and (2) analyze the precipitates by EDX or EELS would the best way to identify them. Stephane Nizets nizets2@yahoo.com Thu Jul 10

Many thanks to those of you who gave advice regarding black

precipitate in sperm preparations for TEM. I am now fairly certain that the problem occurred because of inadequate washes between pre & post fixation. The sperm pellet was very tiny and easily disturbed - washing such a sample in an Eppendorf is not easy! Some other tissue I prepared a few days following the sperm preparation is fine with no trace of precipitate at all. We are going to try high pressure freezing and freeze substitution with the sperm. Ursula Potter u.j.potter@bath.ac.uk Mon Jul 21

SPECIMEN PREPARATION - TEM stabilization layer for delicate polymers

I'm trying to stabilize some very delicate polymers for low-dose TEM imaging (100-1000e/A). These polymers cannot be rewet, so I can't put them in solution and plunge freeze. I'd like to deposit a stabilization layer such as carbon, but I don't want to damage the polymers by depositing 4000k atoms all over them (heat/velocity). I've heard that using indirect arc-evaporation and simply increasing the pressure in the chamber forms a super low dense film morphology, which wouldn't be very stabilizing. We're thinking maybe PLD is the most controllable. Does anyone know of a way to deposit a tight encasing layer gently? Stanly Kemish j.whitt31@gmail.com Sun Jul 6

Have you looked into plasma-enhanced chemical vapor deposition? There are several configurations for generating the reactive vapor, some of which may be gentler than others. One method that admits naphthalene vapor through a column with inductively coupled plasma generation should keep the plasma away from your sample. In some experiments, I reversed the polarity of a DC sputter coater and while I got nice films with naphthlene on mica, the plasma at the surface of polymer films was too much and they broke; maybe the remote generation method would work more gently. It looks a little less complicated and expensive than the PLD method. Dale Callaham dac@research.umass.edu Mon Jul 7

I have two ideas for you: You can cool the specimen to LN_2 temperature without plunge freezing—just put it in a cryostage, put the stage into the EM, then cool the stage. Most of the heat transmitted to the specimen from a C arc comes from the photons, not the C atoms, so you can turn the arc on only long enough to get a very thin C layer, then let everything cool down (Check that the vacuum returns to what it had been.), and repeat until a sufficiently thick C layer has been built up. Bill Tivol tivol@caltech.edu Wed Jul 9

SPECIMEN PREPARATION – SEM of small round samples with charging

I'm having problems with a yeast prep for SEM. The cells are grown in liquid, fixed with glutaraldehyde followed by osmium. For mounting the yeasts, I have put them onto Millipore filters (0.22 µm, Type HA, native or carbon or sputtered both sides), adsorbed to carbon-coated glass treated with polylysine or polyethyleneimine (0.1%). I get nicely stuck cells, good distributions without pile-ups. I stuck them to polyethyleneimine-treated aluminum foil (subsequently mounted via carbon tape) to make sure the substrate conductivity is not the issue and I get nice monolayers of cells. The cells have been critical-point dried or dried from HMDS. I sputter coat as I normally do (2-3 min at 5 mA, 2.2 kV in argon - should give ~12-16 nm Au:Pd), but the result in all cases is most cells charging badly (5 kv, 55 µA load current) in conditions that most biological samples I work with do not. I've experienced this with pollen grains and other rounded specimens and it looks like "textbook" images that JEOL uses of toner particles to illustrate the limited contact mounting issue - these are all samples that make little contact with the substrate; and in my case I can't press them into a carbon tape. See Figs 24 and 25, http://www.jeol.com/sem/docs/sem_guide/guide.pdf. I'm assuming that it is the limited contact area that is mostly shaded in the sputtering process that is causing my problem. Although sputtering does fairly well getting the sides of things, is this one of the cases where rotation while sputtering might be a help? Dale Callaham dac@research.umass.edu Wed Jun 25

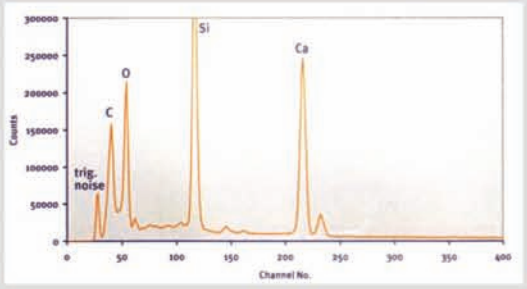
Tilt and rotation. Perpendicular coating can't cover the sides of 3D specimens very well or at all. Gary Gaugler gary@gaugler.com Wed Jun 25

Seems like you've done most everything I would do for cells like that for SEM, and I don't recall having any serious charging problems as you describe. It could be a contact issue, and gyrational tilt-a-whirl coating in a vacuum evaporator might eliminate the charging. However, one option to consider, is the double osmium fix called "OTO". You apply the first osmium fix as usual (in distilled water), then rinse that out (distilled water) and add the "T", thiocarbohydrazide (TCH), usually dissolved by heating to saturated solution, then cooled and filtered before applying to the sample. Then rinse that out (distilled water) and apply a second osmium fix. Rinse that (distilled water) and proceed to dehydrate, etc. as usual. The TCH acts as a mordant to bind in more osmium the second time, enough so that you get a lot of conductivity doped into the cells which results in no charging under the beam. Years ago, I had horrible charging problems looking at fungal colonies grown on agar. No matter how much gold I evaporated on, still charged. The OTO method totally eliminated the charging. I can send you protocol and reference off-line if you would like to try this. Gilbert Ahlstrand ahlst007@umn.edu Wed Jun 25

A common problem that one of our SEM students, Amanda Best, had with pollen grains. I showed her how to get around the lack of coating on the bottom** using cardboard, which takes forever to degas, and she came up with a nice solution. Microscopy Today, July 2007, pg52 ("Microscopy 101"), SEM stub holders for sputter coating at 90 [degrees] tilt". The holders are modified shelf brackets, easily made. This is now a routine procedure for a couple of labs here. If you don't have the issue, it can be downloaded as a pdf from the MT website, http://www.microscopy-today.com **This was addressed some years ago by Mary Fletcher at UBC, but I forget the print reference. Sorry Mary. Philip Oshel oshel1pe@cmich.edu Wed Jun 25

These are the most common solutions when having problems with conductivity - 1. Incomplete coating - rectified by tilting the specimen ± 45° by multiple coats if that is the only way. 2. Run at a low kV (that is what you are doing but could you go lower?) 3. Reduce the spot size beyond what is normal for the magnifications in use (less electrons hit the sample so less need to bleed away to earth) 4. Lower the emission current (less electrons hit the sample so less need to bleed away to earth) 5. Put on a slight positive tilt (increases the BSE contribution to the signal and BSE are less effected by charge) 6. If you have a dual SE detector system use the lower detector (increases the BSE contribution to the signal and BSE are less effected by charge). Steve Chapman protrain@emcourses.com Wed Jun 25

SDD
Silicon Drift Detector

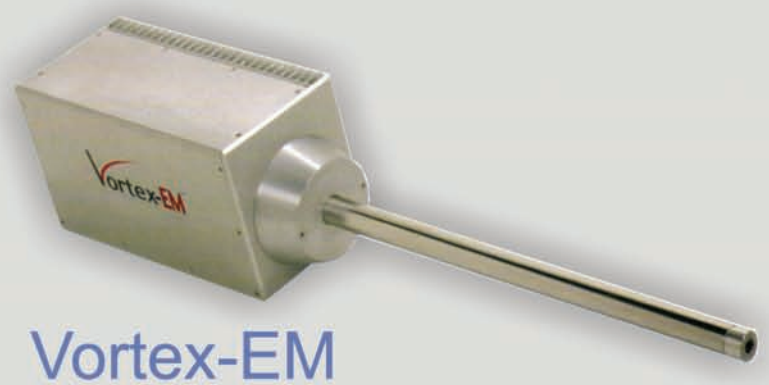


Typical light element spectrum

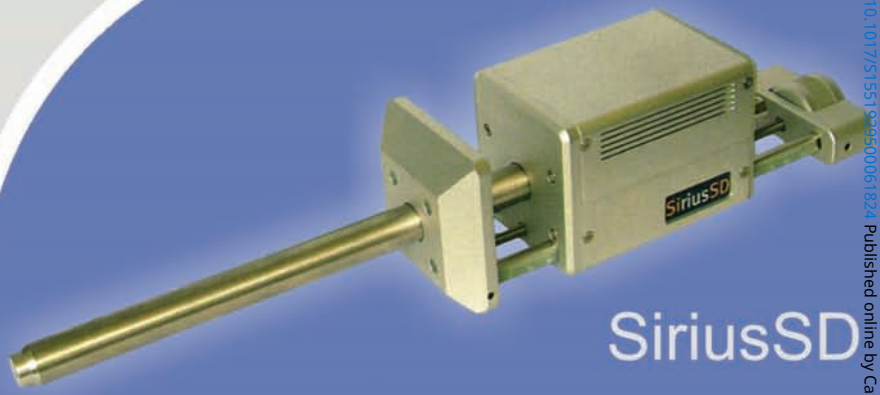
Energy Resolution (FWHM) @ 5.9 keV < 126eV
Crystal active area: 10-30 mm²
Crystal thickness: 450 µm
Window: Ultra Thin Window

YOUR CHOICE

50mm²



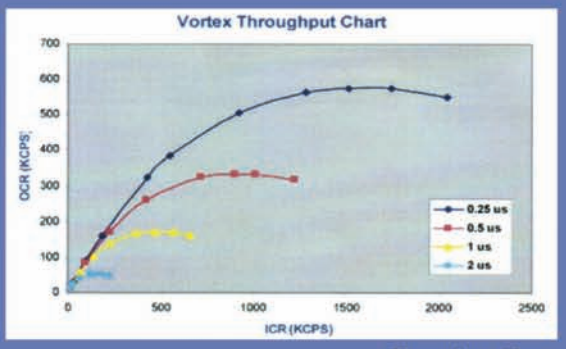
Vortex-EM



SiriusSD

126eV

Energy Resolution (FWHM) @ 5.9 keV < 133eV
Crystal active area: 50 mm2 (nominal)
Crystal thickness: 350 µm - 400 µm
Window: Ultra Thin Window



Throughput Chart

SDD
Silicon Drift Detector

IXRF Systems

IXRF Systems, IncHouston, TX77062 Ph: 281-286-6485 F: 281-286-2660

I watch Dennis Kunkel (http://www.denniskunkel.com) do this all the time, the cheap and easy way. He sputter coats from the top, then he lies the stubs on their sides in the coater and coats, then he rotates them a third of a turn and coats, then another third of a turn and coat, then another... and may finish off with another coating from the top if stuff is really piled up. It takes a little while, but doesn't require a tilt-and-rotate stage. We use the pin stubs, so they lie at about 50 degrees, I guess. If you have cylinders, they will lie at 90 degrees. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Wed Jun 25

SPECIMEN PREPARATION - small, irreplaceable mineral grains for analysis

I spend considerable amount of my time analyzing single grains of very rare minerals. Typically there is very little material available for probe analysis so I have to work with grains in the 100 - 200 μm range. When I mount these grains in epoxy, I want to grind as little material as possible before I polish. At present, I smear a very thin layer of Apiezon grease on a glass slide, get the grains on the grease and cover them with one drop of epoxy. When this first drop sets I drop a plastic mounting ring over the grains and fill as normal with epoxy. When the epoxy sets I can usually detach the glass from the ring and hopefully have the grain exposed on the surface of the mount ready for polishing. In general, this works, but I'm having some problems with the grease preventing the epoxy from sticking to the grain (This probably depends on the surface properties of the mineral). The surface of the epoxy is also wrinkled with this technique, making polishing difficult. Does anyone have another technique that would work for this type of preparation? A substitute for the grease that would hold onto the mineral grains and allow removal of the glass slide would be really nice. Glenn Poirier gpoirier@mus-nature.ca Wed Jun 25

When dealing with similar grains in our lab, we previously followed a procedure much like yours. Now, though, we drill holes in a disk made from a material harder than the set epoxy, usually acrylic or polycarbonate or occasionally even stainless steel. This provides some additional protection during polishing so that material doesn't wear away as quickly. The grains are held at the bottom of the holes using ordinary plastic ("invisible") tape. Our tape-epoxy combination doesn't seem to cause any setting problems—no guarantees for anyone else's tape or epoxy. Any bubbles in the epoxy are removed by pumping down the mounts in a vacuum impregnator and, if necessary, a syringe with a hypodermic needle. This works pretty well for us—your mileage may vary. Ellery E. Frahm frah0010@umn.edu Wed Jun 25

Try a dab of Loctite 4305 (UV curing) instead of grease. It is very low viscosity and will fully wet the sample. Also, it will fully adhere to your vacuum-impregnated epoxy, such as Struers Specifix-20, Buehlers Epo-Thin, etc.... Another way is to dab a bit of Gatan's G-1 and cure at 100°. Or, use an equivalent brand. J. Quinn jquinn@www.matscieng.sunysb.edu Wed Jun 25

Something to try without the need for any grease: use a silicone rubber base rather than a glass slide. Epoxy doesn't stick to it and it has the advantage of being slightly tacky so your valuable small sample is harder to lose. In semiconductor manufacturing there are a variety of types and sizes sold under the name of 'Gel-Pak' which are used to transport things like diced up lasers - which can be as small as 100 x 200 μm. I use them as a work surface for TEM specimen prep since they are clean, solvent- and epoxy- resistant, and small fragments of material stay where you put them. You can also get silicone rubber inserts for Petri dishes from microscopy houses. Richard Beanland contact@integrityscientific.com Wed Jun 25

There have been several really good suggestions already. One technique I have used for embedding thick epoxy sections of biological material for re-sectioning for TEM examination involves using a release agent on the glass slide. One such product is available from EMS. You can find it in their online catalog at: http://www.emsdiasum.com/microscopy/products/preparation/coating.aspx#70880. You coat a clean slide with this liquid, let it dry, then mount your sample onto it the same way you are now on your coated slide. Once the resin is cured, you can soak the prep in water and the release agent dissolves and you have a clean flat surface to polish. I don't know whether your particles would stay where you want them, though, since the surface is dry and hard. John Chandler jpchandl@mines.edu Wed Jun 25

I've used Rain-X, which is available at most hardware or automotive stores, for this very type of thing. You treat the slide with it and cured resin pops off very easily, no soaking required. It's cheap and a little bit goes a very long way. Jay Campbell microtomy@gmail.com Wed Jun 25

SPECIMEN PREPARATION - making support films for slotted grids

I am trying to make my own 1 x 2mm slotted grids with a support film. It's getting quite expensive to buying them. I have tried a few things (1% Formvar, 1% nitrocellulose , both with carbon) but they do not seem to be stable enough. Does anyone have any "special recipe" they might want to pass on? Margaret E. Bisher mbisher@princeton.edu Thu Aug 14

These days, I find that 1% Formvar or Butvar is just not strong enough. Try 2-3%, instead. Nitrocellulose definitely should be at least 2%. In addition, I always lay down a thin carbon film if I notice that the films are breaking or drifting. Carbon deposition can be done either right after making the windows OR, even better, after the stained sections are on the films. Then, they are rock solid and never prone to charging. John J. Bozzola bozzola@siu.edu Thu Aug 14

You may have some luck by making a fresh solution from the resin. I had some issues with an older solution and was informed that light causes the dichloroethane solvent to form HCl. This damages the Formvar polymer. I made a fresh solution and kept it in a dark bottle in dark cabinet and the solution lasted well for a couple of years. This is a 0.25% solution on 200 mesh grids. Hendrik O. Colijn colijn.1@osu.edu Thu Aug 14

I have had only limited experience with slot grids, but those I have prepared I made the film out of 0.5% Formvar. It is important to use freshly prepared Formvar, since the films seem to get more brittle as the Formvar solution sits. I always coat with C after picking up the Formvar film on the grid, and I also coat again with C after the sections have been placed on the grid. Formvar is very susceptible to charging, so never look at the grid without having the objective aperture in place—the electrons backscattered from the Pt will neutralize the charge build-up due to loss of electrons from secondary electron production. The only time you should disregard this rule is when you are scanning the grid at low magnification,

and then you should use a high spot size number and widely spread beam—the lowest possible dose rate. After looking at sections at ~100×, I would put in the aperture and go immediately to the lowest magnification in M or SA mode (~1000× on my instruments). Bill Tivol tivol@caltech.edu Thu Aug 14

MICROTOMY - cleaning a diamond knife

We encountered a persistent scratch problem when we're cutting resin blocks with our diamond knife. Although we did all standard cleaning procedures, by using its original cleaner with absolute ethanol, we couldn't solve it. Any comments? Necat Yilmaz nyilmaz@mersin.edu.tr Mon Aug 4

You don't say how old your knife is or how heavily used. I am assuming that you are cutting biological samples as you mention resin. Scratches do eventually appear in diamond cut sections especially if many different users use a single diamond for different materials.

If the scratches have appeared over time, it might just be normal wear and tear but it might also be gritty or hard samples or centrifuged pellets that can accumulate tiny fragments of glass or deposits that could damage a diamond when sectioned. There are special cleaning fluids that you could try in case it's just a persistent particle stuck to the knife edge, but you need to be careful because some solvents will attack the adhesive mount of the diamond. If you do use such a fluid make sure it's for diamond knife cleaning and follow the instructions carefully. My regime with diamonds has been to cut as far to the left on the knife as possible and if it eventually scratches my sections move to the right until they are undamaged or

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I run out of knife. This works best for wide cutting-edge diamonds and small block faces. If all else fails you might have to consider re-sharpening, a new diamond or glass knives. Malcolm Haswell malcolm.haswell@sunderland.ac.uk Mon Aug 4

Is the scratch always at the same place relative to the knife? If so, treat the knife. Is the scratch always at the same place relative to the block? If so, the problem comes from the block. I have noticed that some irregularities in resin curing may cause scratches (with Epon). Cleaning the knife edge with Styropor sticks dipped in alcohol should clean it pretty well. Usually it is my "last resort" and always worked. If you suspect the edge to be damaged, try to inspect it with a microscope (don't try to insert it in the TEM though). Stephane Nizets nizets2@yahoo.com Tue Aug 5

IMMUNOCYTOCHEMISTRY – re-using antibodies

I was wondering if you could please give me some pointers on re-using antibodies. I have an antibody that is a gift and in a very limited amount. I would like to run several slides sets with the same antibody solution made over a period of 4-5 days. Do you prepare your primary in the blocking solution with this? Do you add sodium azide, if so, how much? I would appreciate any advice based on your IHC experiences. This will be used on cryo sections and immunofluorescence application. Marti martimor@nmsu.edu Mon Aug 4

I have already seen antibodies reused several times for Western blotting, but I never heard somebody re-using them for IHC. I don't know why though, but there may be some rationale. However, I can give you a trick to limit the volume used for the reaction. Usually people use a big drop (100-150 µl) on a slide for the incubation. I prepare a 30-50 µl (30 µl should do) drop on a Parafilm and invert a coverslip on it. To wash you just use a syringe and infiltrate the washing buffer under the coverslip. It avoids having to move your sample and risking damage to it or letting it fall. Given the very limited volume present under the coverslip, you must incubate in a humid atmosphere (which is anyway always advised). This is for 18 ×18 coverslips. Stephane Nizets nizets2@yahoo.com Tue Aug 5

Just to follow up on Stephane's idea - we also use the coverslip approach but we use the pre-made coverslips designed for this (e.g., "LifterSlips" but alternative versions available) available from most microscopy supply outlets. I routinely used the 22 × 30 mm coverslips which require 15.6 µl to cover the surface but their 18 × 18 mm coverslip only requires 7.6 µl. We incubate overnight in a humidity chamber and then add a drop of buffer to the edge of the coverslip for 5 sec and then rinse it off into a beaker with more buffer. We recover the coverslips and wash them for re-use so it is quite economical. Tom Phillips phillipst@missouri.edu Tue Aug 5

In my old lab we routinely re-used primary antibodies for IHC 1 or 2 times with overnight incubation times, normally in the refrigerator. Between the incubations you store the antibody solution in the fridge if you use it again during the next 24h, if not you put it in the freezer. We never used sodium azide in the antibody solution but our specimen were routinely stored in sodium azide before IHC so there may have been some residues in them although we always washed them several times. However, it does not work for all antibodies—you have to test it. In addition, even for some of the antibodies that we routinely re-used it did not always work. You have to be careful that you e.g. do not get particles from your specimen into your antibody solution if you rescue it from a sample after incubation. So you won`t re-use an antibody on a sample that is very important or rare, let`s say if the sample is the limited resource. If not the sample is the limited resource but the antibody (as eventually in Mortimer`s case): what do you lose by re-using the antibody, just try it. If both, sample and antibody, are limited, you may ask the source of the antibody whether they have any experience in re-using it. In the end, you have to decide whether you take the risk of eventually losing a valuable sample. Christian Liebig c.liebig@imperial.ac.uk Tue Aug 5