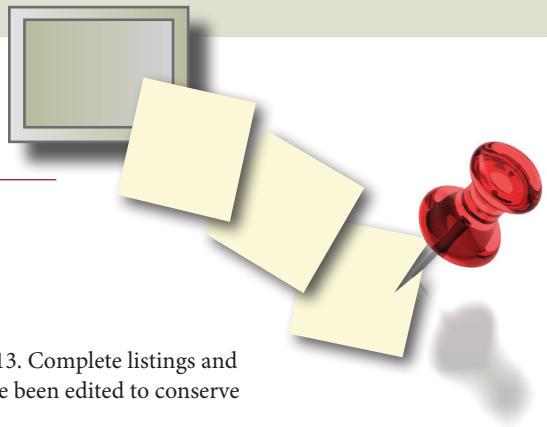

NetNotes

Edited by Thomas E. Phillips
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Selected postings from the Microscopy Listserver from September 1, 2013 to October 31, 2013. 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:
embedding cell cultures

We have been doing TEM for years on cells growing on cell culture plates by doing all of the processing and embedding directly in the dish. We leave a little film of resin behind from the final straight resin change and then invert BEEM capsules filled with resin onto the dish. After polymerization of 24 hours we then let them cool and "pop off" the BEEM capsules bringing the cell layer with it. We have found through the years that certain cell culture plates will not stand up to the chemicals in the resin and you will get kind of a cloudy mess. We've also learned that any alcohol must be thoroughly removed before adding any resin, as the combination seems to really eat away at the plastic in the culture dishes. For the same reason we don't do a propylene oxide wash for this procedure. However, through trial and error we have been able to keep a list compiled of which plates work with which resins and have had success using this method. We still routinely check the plates with the resin we intend to use before we do a real processing to make sure that nothing has changed since the last time we did it. Using this method we have always been able to get a resin surface where 50-75 % is usable for sectioning. There is almost always a small area where you might get some plastic that comes off with the resin. We have always been able to tell if the embedding worked before even popping the BEEM capsules off by just looking at the polymerized resin. If it is clear the capsules will pop off mostly clean, if the resin is cloudy the procedure is a bust. However, recently we have been getting way too many situations where the resin is perfectly clear yet the BEEM capsules bring up a fine layer of plastic with them that makes sectioning impossible. This seems to be happening with different resins and plates from different manufacturers. Does anyone else use this procedure for TEM of cultured cells and if so are there tips or tricks that would help us? I know you can scrape and embed in agar, etc. but I would rather continue doing things the way we have in the past if possible. ***Jim Begley jgbegl2@email.uky.edu Mon Sep 30***

Nearly all tissue culture dishes and flasks are made of polystyrene and I have had good luck using my main Epon component from Ladd. It is called LX-112. It works as well as original Epon812 in the dishes. The NMA, DDSA, and DMP-30 I have not had a problem in using from different suppliers. I believe that the Epon from SPI (and maybe others) will also work, but I personally have not used it. Electron Microscopy Sciences states in their catalogue that "Embed 812 may cause etching on selected plastics." It melts the surface of TC dishes from at least four suppliers that I had checked out many years back when I ran out of Shell's Epon812. I had informed EMS of the problem and hence the warning. I do use their other components. Most cells are happy growing on Permanox dishes. They are larger than I'd wish, being 60×15 mm, and are expensive as dishes go, but one can use any embedding chemicals as well as propylene oxide and they stay very clear and smooth. The BEEM capsules come off very easily. I requested a few years ago for the company to make small dishes out of Permanox, but I'd need to order a run of many thousands of cases! When it is necessary to limit the growing area as in pre-embedding immune work, we use the two well Permanox slides. I can fit either two normal-sized capsules in each well or three small ones. I have sliced off the end of the capsules and filled them from the top instead of your method of filling the capsule first. I usually put the capsules into the chamber then cure overnight before the fill and label steps so that the Epon does not leak out and fill the whole chamber. Within the small space of the chamber your way may not work too well. Note: the sides of the wells are polystyrene so do not use Propylene oxide, and they will melt if not using the LX-112 mentioned above. Patricia Connelly connellyps@nhlbi.nih.gov Mon Sep 30

Like Pat Connelly, I use LX112 from Ladd as well. I embed in culture dishes, without the BEEM capsules. After polymerization I just cut out the cells of interest in a shape that fits in the ultramicrotome, or I Krazy Glue them to blocks. To separate the polymerized LX112 from the dish I either try to peel it off while the dish is still hot from the embedding oven, or I go the other route and try to separate them after dipping in liquid nitrogen (sometimes by throwing them against the wall). It's not always pretty, but I've never failed to get the plastic off. Do not use propylene oxide! But LX112 in ethanol does not melt the dish or make for foggy resin. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Mon Sep 30

Specimen Preparation:
Formvar grids

Call me naive, but I have a question about getting Formvar films to release. Students here are going through the initiation process of making coated grids. Sometimes it works; sometimes it does not, even for me. I was looking over a Dear Abbe column in a recent Microscopy Today that addressed this same question. It suggested a few tricks I had never heard of. One that was intriguing was to lick the slide first. Now I had never heard of that, but am getting desperate enough to try it. I know some of our students might read this advice from Abbe and try it without clearing it, is it for real? Seriously, how about an update on any tips or tricks on getting that Formvar off the slide every time. ***Jonathan Krupp jkrupp@deltacollege.edu Wed Sep 25***

You have to sacrifice an ingot of osmium to the goddess of adhesion… Don't laugh but here is my, highly reliable if not completely perfect, superstitious ritual. Obtain Ross Optical Lens Tissue. This is important, accept no substitutes. Take a piece of lens tissue and vigorously rub the slide surface to be coated. To make it less stressful, I rub both sides of the slide so I don't have to keep track. I typically count ten back and forths (20 each way). Then flip the slide and do another 10. Then dip in Formvar, let dry, score, and float. Having written this down, no doubt the next time I try, it won't work. But up until then, this method has absolutely lowered my stress level for this task. Tobias Baskin baskin@bio.umass.edu Wed Sep 25

We went through this same issue a few years ago with our TEM class: cleaning slides, not cleaning slides, clean but leave a bit of "soapiness", breathing on the coated slides, nose grease, squashed cockroach goo (hey, it's cheap), etc., and eventually settled on one thing: Grafco Cat # 3703-2P slides with clear/ground edges, Graham-Field Health Products www.grahamfield.com. They're cheap and reliably release the Formvar film. Found them in the Intro Biol lab supplies, because they're cheap. The other brands & catalog number slides we tried had the same issues you're having. Phil Oshel oshel1pe@cmich.edu Wed Sep 25

Because casting films onto microscope slides is a "black art", I use freshly leaved mica sheets for both carbon and Formvar films. I just dip the mica into the solution and let it dry. It is necessary to take a razor to score the edges so that the water can penetrate under the film but once it does, the films lift right off... no black magic or voodoo chants required! Henk Colijn colijn.1@osu.edu Wed Sep 25

Not my reply, but forwarded for an industrial microscopist who must remain anonymous: "When I made my own films many years ago, I used the bevel-edge slides you described. An important step is to etch a rectangle in the Formvar film on the slide prior to releasing it onto the surface of the water. I did this by using a sharp razor blade and cutting several millimeters inside the edges of the slide. Others in my lab scraped the edge of the slide with a razor blade to promote release." Aren't lawyers fun? A person can't reveal their name for a post like this … Philip Oshel oshel1pe@cmich.edu Wed Sep 25

In a previous life we would meticulously clean our slides (which "had" to be a certain brand, but I don't remember which one), then place them in racks in a vacuum evaporator with a big bell jar. We would use a tungsten coil and place a chunk of a soapy substance called Victawet in the basket, then pump down and heat up the coil until all the Victawet evaporated. Then we took out the coated slides, which looked like they had a hazy film on them, and box them up until needed. Before dipping the slides in Formvar solution we would polish them vigorously again. The Victawet seemed to act like a release agent, but sometimes even that didn't work. We had this long elaborate ritual with uneven results. Years later I mentioned this to Kent MacDonald and he proceeded to demonstrate his method, which was a no-frills, straightforward "pick a slide out of the box, wipe it off, dip it in Formvar and dip it in water" sequence. Beautiful films that released the first time. I seem to recall that he polished the slide with his shirt, but I'm not sure anymore, and I beg his forgiveness if I have misremembered that part! Motto: Formvar film release appears to be in the hands of the gods (and EM legends like Kent). Randy Tindall tindallr@missouri.edu Wed Sep 25

Well, the fates clearly have it in Formvar. Moments after posting about the miraculous Ross Optical Lens Tissue (and I use the word miraculous in all seriousness), I was informed by Ted Pella (who sold me my treasured box of Ross) that these tissues are no longer made. He tells me that the replacement product their company found has been field tested in EM labs and found satisfactory. But given the notorious quirkiness of Formvar, one has to wonder… One good thing from Ted Pella about the replacement—the new box really does have 1000 tissues--apparently the Ross box claimed 1000 but actually had only 800 (they counted!). And despite this dodge Ross went out of business. Perhaps a warning? Tobias Baskin baskin@bio.umass.edu Wed Sep 25

Specimen Preparation:
embedding Media for STEM/SEM EDS mapping

I am recently looking into STEM EDS mapping of some ultramicrotomed samples. The problem is that we have always used Epofix and Specifix for observing such samples in the TEM but they don't seem to be too stable in the STEM. I have heard that acrylates are much better but have no experience with them. Does anyone know resin that can be stable enough under EDS/WDS mapping conditions? Has anyone done any mapping using aberration corrected STEM probe? Since we plan to do ultramicrotomy with the samples I think I have to use a hard epoxy. Am I correct? ***Ram Chandra ramchandra.t@gmail.com Sun Sep 8***

Have you considered putting a thin carbon coat on your samples? I've found that sections are much more resilient to the e-beam with a carbon coating. My experience is that uncoated epoxy sections "blow-up" the moment I focus the e-beam on them. I assume that the carbon primarily provides a conductive path reducing charge build-up. So whether the sample blows apart to electrostatic forces or decomposition of the epoxy, the carbon makes a world of difference. Henk Colijn colijn.1@osu.edu Sun Sep 8

Try grids with carbon film. Sections should behave nicely. Vladimir Dusevich dusevichv@umkc.edu Mon Sep 9

Thank you very much for responding to my question. Based on the Input I have received, I think LR White and Spurr equivalent would be the best choice. I will also coat them with carbon to see what happens. The idea of mixing nanotubes is also very interesting and out of the box but maybe complicated for the beginning. I do wonder if carbon coating a sample causes contamination issues in an aberration corrected microscope. Ram Chandra ramchandra.t@gmail.com Thu Sep 12

Regarding contamination from the C coating. We generally don't see any. The amorphous C coating is pretty tightly bonded. Most of the contamination we see seems to be due to small organic molecules which can rapidly diffuse across the sample under the influence of the e-field from the beam. If you do see contamination, I would suspect it comes from unreacted epoxy monomers. Henk Colijn colijn.1@osu.edu Thu Sep 12

Specimen Preparation:
staining of agarose for TEM embedding

I have some users who have very few precious bacterial samples. When they tried enrobing them in agarose for fixation and resin embedding, not only did the cells get a little too dispersed (I told them they need to practice), but their tiny agar blocks were nearly invisible in the unpolymerized and polymerized resin, and they lost some. I remember in the dark past that the agarose could be stained with something that would remain through the processing and into the resin, possible something blue. I cannot find that reference now, and even with a very specific wording, Google comes up with 8,080,000 results in 0.40 seconds. Any favorite protocols gratefully entertained! ***Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Fri Sep 13***

Have you tried toluidine blue? It's metachromatic for mucopolysaccharides. But as it is basic, it would have to be used after fixation and any uranyl acetate en bloc stain. It should bind well enough to the agarose to still be there when the resin steps are reached. If the toluidine blue does want to come out of the agarose, a trick to try is ammonium molybdate, 5% aqueous (must not be cloudy) for 5 min following the toluidine blue, then rinse in "running tap water" (approximate this) for 2-3 minutes. The molybdate acts as a mordant, and after this treatment, the toluidine blue won't come out in water or alcohol. Note: you may have to use acidified toluidine blue to use molybdate. (Kiernan, "Histological and Histochemical Methods: Theory and Practice" 3rd edition.) Phil Oshel oshel1pe@cmich.edu Mon Sep 16

Back in the day, I did this with fast green. I made a saturated solution in 100% ethanol, and then would add a microliter per sample vial (say 1 mL or so of ethanol) at the 100% stage. To be fair, I was infiltrating in butyl methyl methacrylate not epoxy. It is possible that the color would be more strongly extracted by those resins. But the

fast green did fine for the agarose in the methacrylate. Tobias Baskin baskin@bio.umass.edu Mon Sep 16

We routinely use 2% potassium permanganate ($KMnO_4$) to fix agarose embedded yeast cells, because osmium does not penetrate the yeast cell wall. One "by-product" of potassium permanganate fixation is that it completely turns the cells, and the agarose to black, making the block visible for sectioning. I assume (never tried myself) osmium will do the same thing, except that the osmium won't penetrate the bacterial cell wall either. For the sake of "staining" the agarose, it might worth to try. If you are doing immuno-gold labeling, however, that won't work! What we do is to try to identify the cells under a dissecting microscope, mark them with a sharpie and go from there. Zhaojie Zhang zzhang@uwyo.edu Mon Sep 16

When I send my blocks to a campus facility for paraffin processing and embedding, I need to mark one of the edges so they can know the orientation I want. They gave me a little vial of tattoo ink pigment in powder form. I usually add it to the connective tissue side opposite the epithelial surface I am interested in using a wooden applicator. The big pigment particles adhere pretty well and easy to see. I suspect you could add a couple of tiny particles from it to your agarose and be able to see it in a stereoscope. I haven't tried this for EM blocks but suspect it would work. This might minimize the chance a stain would interact with your bacteria. Tom Phillips phillipst@missouri.edu Mon Sep 16

Specimen Preparation:
Thiery silver proteinate stain

We're having a problem using a variation of the Thiery technique in an attempt to stain glycogen on thin sections for TEM. The staining is actually working OK but we are getting very bad silver protein precipitation on the section surface in the form of web-like masses as well as aggregates of discrete colloid-spheres. We are using the silver proteinate at 1% aqueous and have tried dissolving 24 hr in advance, centrifuging at max speed in tabletop centrifuge, and 0.2 um polymer filtration through syringe prior to incubation (all kept dark). Extensive washing with di-water following silver incubation does not help. Does silver proteinate have a definite shelf life beyond which it produces these sorts of problems? Our dry silver reagent is several years old but has been kept wrapped in foil at room temp and looks good, no moisture or clumping in the vial. ***David Lowry dlowry@asu.edu Wed Oct 30***

I have no experience with this technique so cannot help you with it. However, you might consider an alternative technique next time this comes up. This is the PATO technique utilizing ruthenium red to stain polysaccharides. It has worked very well for us in the past identifying glycogen deposits in cyanobacteria (see references below). You can download the technique from my website. J.S. Hanker et al., "Osmophilic Reagents: New Cytochemical Principle for Light and Electron Microscopy," *Science* 146 (1964) 1039–43. Sherman, Debra M. and Louis A. Sherman, "Effect of iron deficiency and iron restoration on ultrastructure of *Anacystis nidulans*," *J. Bacteriol.* 156 (1983) 393–401. Debra Sherman dsherman@purdue.edu Wed Oct 30

Immunocytochemistry:
negative staining

I need to do some negative staining of antibodies. My uranyl formate is long dead, and the uranyl acetate is a bit grainy. I have seen references to but never tried sodium silicotungstate, although it seems to come recommended for its fine grain size. I also see references to methylamine tungstate, and I see EMS has sodium tungstate. I have not used any of these, only PTA, plus ammonium molybdate. Do any of you have any recommendations for a fine-grained negative stain for e.g., antibodies and proteins of similar size? ***Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Mon Sep 9***

I want to thank the people who replied. A few pointed out some new (to me) stains available, including Blue Platinum from IBI Labs, NanoVan from Nanoprobes, and a non-radioactive uranyl acetate replacement, UAR_EMS, from EMS. Other stains I have not personally tried include sodium silicotunsgtate and methylamine tungstate. Shipping radioactive or hazardous chemicals to Hawaii is prohibitively expensive, so I'm looking for an alternative to the really old, photodegraded uranyl formate I had that had a fine grain size and high contrast. Recommendations from people generally insisted that the uranyl stains need to be fresh and my stains were a bit old. In addition, I do not have any pure carbon films right now. I used to make them by evaporating onto mica and stripping them off, but my evaporator has a leak. I have to admit that I have not repaired it because the leak is exactly the right amount for glow discharging grids, so I have had little motivation to fix it. I have not had much success with commercial carbon-with-removable-Formvar grids. You may detect some laziness on my part, so I will clean up my act and try again! To clarify what I am trying to do, a researcher has some evidence that some antibodies from a sea creature that will for now be unnamed (not sure how much I can disclose) have a strange configuration. In addition to the typical "Y" shape, there is probably another smaller Y complex sticking off to one side. I can see pretty well the larger protein loops, but I need to be able to resolve some smaller structures. A smaller stain grain size would help plus, obviously, cleaning up some background. My lazy technique has been adequate for all these giant marine viruses we look at, but I need to refine this to see smaller structures. The sample is 1 µg/ml in sodium borate pH 8.0. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Wed Sep 11

I am sure you have more than one argument to explain your summer laziness in a beautiful place like Hawaii. By reading your message about your loch-Hawaiiness monster I remembered an interesting instrument called low voltage electron microscope (LVEM). Perhaps it is the right application for this instrument, because your main problem is the lack of contrast and the difficulty of developing a contrasting methodology. Due to the low voltage of the LVEM, perhaps you can see your protein complex without contrasting at all. I'll bet Delong would be interested to find a new application for their instrument if you care to contact them. Otherwise I wanted to cite shadowing as an alternative to negative contrasting. Stephane Nizets nizets2@yahoo.com Thu Sep 12

Image Processing:
montage viewer

Can somebody suggest a simple compact off-line program to view large images that result from montages of many smaller images? The key functionality required: easy zoom-unzoom and measurement of the distance between given points. ***Pavel Potapov pavel.potapov@globalfoundries.com Mon Sep 9***

With ImageJ 1.47h and Windows 7 I can open a 0.5 GB image with no problem and make measurements. It is just necessary to set the memory buffer large enough in the options for the program. I don't know the maximum file size possible, but you can play with it. Larry Scipioni les@zsgenetics.com Mon Sep 9

Image Processing:
colorization of SEM images

We would like to colorize SEM images of insects to make a visual impact for an art piece for framing. We have taken both a secondary

electron image and a separate back scattered image of the same field of view. We have mucked around with these images in Photoshop trying to merge them together to produce brighter highlights, changing the hue and colors but the result has been not very appealing. We have also tried colorizing just using the secondary electron image but the result has been flat looking, with less highlights. Does anyone know how to do this successfully, any web sites, you tube videos explaining how to do this? Or are there any software packages available to do this more easily with better results. ***Sandra Crameri sandra.crameri@csiro.au Tue Sep 17***

As others from the list answered, there are two principal ways to get color in your SEM images: - make good black and white images and add a lot of "Photoshop magic" to bring color to the specimen - use multiple detectors and perhaps also different kind of detectors (like SE or backscattered…) to get different signals back from the specimen and use these signals to attribute aesthetical colors to the image (and add some Photoshop magic). For example: I use the secondary electrons for getting a very fast separation of parts on specimen with a small interaction volume like hairs on insects or cells or substrate etc. The backscattered detectors (I have three) I use to get surfaces (topography) ad hoc separated in a different color. You can also try to get more different images with different accelerating voltages and the best suited detectors for it… Also distance of the detectors to the specimen can make very different images… There is a PDF from a Mikrokosomos article which describes my work, but only in German language: www.elektronenmikroskopie.info/pdf/Mikrokosmos06-99.pdf (1 MB) I am using a digital image acquisition system from point electronic, www.pointelectronic.de, which can handle up to 8 detectors and scan up to 4 detectors in a resolution of max. 16kx16k pixel. The scanning software has the ability to directly mix the signals from the SEM into a colored image. For the images, have a look at: http://www.electronmicroscopy.info/shop_biology_plants.htm for biologic specimen and http://www.electronmicroscopy.info/shop_materials.htm for some images from material science. Sorry, pages are in parts still in construction. And if you would like to see some very crazy stuff concerning colors in the SEM AND movement, see my nanoflights at www.nanoflight.info. For this project I got the "Technikpreis 2013" from the German Society of Electron Microscopy at the bi-annual meeting three weeks ago. This had been the film for the award: https://vimeo.com/73030307 Stefan Diller stefan.diller@t-online.de Thu Sep 19

You need 3 images for RGB channels or layers. It is easy if you have solid state BSE detector: you can make pictures with just one sector A, another picture with sector B, compositional picture A+B, topographical picture A-B, and, of course, SE picture. So, you will have five pictures and you need just three of them. In Photoshop try adjusting channels (layers) independently before applying operations to the whole image. You can also use the same technique even if you have just one SE image. You can convert it in three images in various ways, for example image as is, inverted image (negative), high contrast image. Some examples of images: https://www.dropbox.com/sh/ddm8z2m4bhfxcqu/Ul4ZIZ4WzO?lst Colored image of table salt was obtained from the single SE image. Vladimir M. Dusevich dusevichv@umkc.edu Thu Sep 19

Microtomy:
diamond knives

We have been using the same brand of diamond knife for 30 years but in the last few years the quality seems to have declined. When we get one that is scratch free it doesn't last more than a few months. Is anyone happy with their knife company? ***Nancy Smythe smythen@musc.edu Wed Sep 25***

A very wise lady (and owner of a large EM supply company) once told me to check my microtomes when I had a similar problem. I had not had them serviced for quite a while. Once they were serviced the problem disappeared. Based on that experience I would recommend you having them serviced every 1-2 years based on usage. It does cost to do so but it is much less than re-sharpening knives. Debra Sherman dsherman@purdue.edu Wed Sep 25

Microscopy:
reference books

I am having difficulty picking out a book for microscopy. I have used George F. Vander Voort's Metallography: Principles and Practice *so far and its text is good but not as in-depth as I am looking for. Thus I was wondering what you could suggest to me a good reference book for both electrical an optical microscopy.* ***Alejanro Hinojos ahinojos2@miners.utep.edu Tue Sep 3***

The usual suspects have already been recommended: Williams & Carter for TEM, Goldstein et al. for SEM/EDS, Bozzola for biological EM, Hibbs and Price & Jerome for confocal light microscopy, and Sawyer and Grubb for polymer microscopy. Any others? Philip Oshel oshel1pe@cmich.edu Tue Sep 3

Instrumentation:
insurance for moving equipment

As we are planning on a move to a new facility, we have been looking at liability insurance coverage for our instruments during the move itself. Although, vendor's insurance covers de-install/re-install, it does not cover any instrumentation insurance during the transport from one location to the other. The question has been raised if we would like to continue insurance coverage (though our university) after installation in the new facility. Our question for list members is...do you have insurance on you major instrumentation through either your facility or a private firm that would cover major issues (flood, storm, fire, etc.) not covered with your standard service contract? In addition, do you have suggestions of any insurance providers specializing in instrumentation insurance during the move? ***Amanda Lawrence alawrence@i2at.msstate.edu Wed Oct 2***

I've installed/moved/relocated 19 electron microscopes here (several microscopes were moved multiple times) at the University of South Florida (I have worked in 2 Departments, relocating labs in both Departments, as well as setting up a Core in another Department). We used national moving companies such as Allied Movers to do the work, using forklifts and pallet jacks to move the instruments from lab to lab, and building to building. We moved one TEM the length of 2 football fields on a forklift, down sidewalks. With moving companies, the company is bonded against loss. If they damage your instrument, they will be responsible for the damages. We always paid our microscope service providers for disassembly and reassembly, and had site surveys performed prior to installation to insure that the instruments would operate at specification when installed. We never purchased additional insurance, and do not carry additional insurance on our instruments. We keep service contracts on our microscopes. We have only (once) suffered one cosmetic scratch to one of the countertops on one of the TEM's. Ed Haller ehaller@health.usf.edu Thu Oct 3

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