

NetNotes

Edited by Thomas E. Phillips

University of Missouri

phillipst@missouri.edu

Selected postings from the Microscopy Listserver from July 1, 2016 to August 31, 2016. 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: glycocalyx protocol for TEM

Does anyone have a good protocol, for imaging using TEM, the glycocalyx in lysosomes in mouse brain tissue (example-Purkinje cells)?
Chip Dye dyel@mail.nih.gov Sat Jul 2

Look at this reference pertaining to reduced osmium and the glycocalyx: <http://www.ncbi.nlm.nih.gov/pubmed/6202662> **Michael Delannoy mdelann1@jhmi.edu Tue Jul 5**

Specimen Preparation: tin balls

Does anyone have a "recipe" for making Tin Balls on carbon? These are useful as test specimens. We wanted to try our hand at making our own Tin Balls. For examples of Tin Ball test specimens I mean see https://www.tedpella.com/calibration_html/SEM_Resolution_Test_Specimens_Tin_on_Carbon.htm or http://www.canemco.com/product-catalog/calibration-standards/18-products/210-sem-calibration-standards#Tin_on_Carbon_Resolution_Test_Specimen.
Dennis Tillman dennis@ridesoft.com Tue Jul 5

Have you thought of polystyrene latex, lots of sizes and as a fluid cheap compared with tin balls? Gives you lots of options for making your own test specimens. **Steve Chapman protrain@emcourses.com Tue Jul 5**

Specimen Preparation: Trichomonas

*A long while back (about a year) I got some very useful advice from this listserv regarding the mounting of Trichomonas samples for SEM. Thank you. I found that applying glutaraldehyde-fixed cells to a poly-L-lysine (or Superfrost+) coated glass slide for 10 minutes, followed by standard SEM processing through osmium, ethanol and HMDS was fine for visualization of the cells for SEM. However, while the control strain (*T. tenax*) processed through perfectly, the test sample (unknown species of dog saliva trichomonas protists) has been a nightmare. The samples can be cultured in variety of media types, protozoan specific media and DMEM-based growth medium. A pH 8.5 or pH 7.2 seems to have little difference in growth rates, and although they apparently prefer a pH of 8.5, they survive adequately at a standard pH 7.2. Initially, we processed the samples through 2.5% glutaraldehyde (GA) in Dulbecco's PBS pH 7.2, washed 1% aqueous osmium, embedded in agar gel, then dehydrated through an ethanol series before embedding in Spurr's resin, or processed for SEM as described above. The samples weren't great - poor ultrastructure and crenelated cell membrane for the TEM, perforated membranes and collapsed appearance for the SEM, but at least they were intact. We have since tried: The same process, but with a buffer pH of 8.4 (samples looked worse) Initial fix in 0.5% GA followed by the same TEM embedding/SEM dehydration procedure but using buffered osmium (worse still) Initial fix in Bouin's fixative*

*followed by the same procedures with buffered osmium (even worse again, as you'd expect) 2.5% or 5% GA in Millonig's buffer, buffered osmium at 4°C throughout (even worse) 2% GA/2% osmium mixture in phosphate buffer, followed by 1% osmium and usual processing all at 4°C (really, really bad - not a single intact cell to be found) We've also tried critically point drying the cells for SEM with no discernible difference - the problem clearly lies before this step It's quite depressing. I feel I'm probably missing something really obvious. The cells that we have observed seem to be collapsed (or sometimes inflated like a balloon), with very poorly intact membranes, and strange lamellar bodies inside the cell with no recognizable organelles (I'm assuming the lamellar bodies are what's remaining of the ultrastructural membranes). I thought it must be the glut affecting the lipid membranes, or maybe some osmotic or ion pump failure after the initial glutaraldehyde fix, but before the osmium stabilized the membranes (hence the Millonig's buffer and the glutaraldehyde/osmium mixture in the final attempt), but every attempt seems to generate results worse than the last. Sometimes I have received the sample with no apparent cells at all, where I think that maybe the cells have disintegrated completely before reaching the EM lab. I might consider that it's the osmium causing the problems, apart from the fact that the cells have sometimes been clearly damaged before osmium exposure. Does anyone have any suggestions of an alternative fixation protocol that may stabilize the cells? Is tannic acid our next option? Higher concentrations of sucrose? **Natalie Allcock nsa2@leicester.ac.uk Tue Jul 5***

The collapsed appearance for SEM and the crenelated cell membrane for TEM suggest that osmolarity of the fixative might be the problem, or at least, part of the problem. The unknown species might just be more sensitive to osmotic pressure. Here is something you can try: Place cells in fixative, and then check cells with a light microscope right away - If you see cytoplasm "leaking" out of cells, the osmolarity is terribly wrong. GA tends to have very high osmolarity, so increase the GA concentration does not help. If that is the case, you might want to start with paraformaldehyde. Once cells are dead and not sensitive to osmotic pressure, then switch to GA. The secondary fixative (osmium) should be less a problem. Have you tried to use the culture medium as the "buffer" for fixative? **Zhaojie Zhang zzhang@uwyo.edu Tue Jul 5**

Microtomy: section wrinkling

*Does anyone know how to prevent section folds in 1 or 2 micron thick sections of plant stem material embedded in Spurr's resin? The sections are fairly large, about 4 × 7 mm, cut on a histo diamond, then placed on water drops on Fisher Superfrost Plus slides and dried on a slide warmer at about 70°C. We get multiple section folds parallel to the short axis of the rectangle, that start at the resin surrounding the tissue and project into the tissue at the center of the block. **Marie Cantino marie.cantino@uconn.edu Tue Aug 2***

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Protochips

You can try cutting the resin away totally so you are only sectioning tissue and don't have that resin/tissue interface. **Susan Van Horn** susan.vanhorn@stonybrook.edu **Mon Aug 8**

Did you try ether on a Q-Tip, hovering over the section on the water drop? **Joachim Siegmund** jsiegmund@7thwavelabs.com **Mon Aug 8**

We have tried this technique with dichloroethylene without success. I think the sections are really too big, but we have gotten some other suggestions that have reduced (but not eliminated) the folds. **Marie Cantino** marie.cantino@uconn.edu **Wed Aug 10**

Thanks to the many people who took time to respond to my question about section folds. Unfortunately the person who is actually testing these tips is on vacation, so I can't comment yet on which ones worked best for us, but since several people requested a summary of responses, I have organized them below:

Fixation and Embedding

- Increase fixation time. If folds are at the centers of the sections there may be a change in tissue fixation (fixed at the periphery but not at center).
- Use long infiltration times, especially if cell walls are thick. For resin-solvent steps and first 100% step, use Spurr's without catalyst overnight followed by two changes of 100% Spurr's with catalyst for a full day.
- Try matching the resin hardness to the tissue hardness by using a different resin mixture.

Trimming

- Make razor blade cuts in the embedding media around the sample then mount sections in the embedding medium to make the cuts disappear (not clear whether the respondent had tried this)
- Make the block face smaller! [The block face seemed very large to me, but I'm an electron microscopist so anything larger than 1 mm seems large. However, it was helpful to get conformation that this is large even for semi-thin sections].
- Trim a trapezoid, not a rectangle.
- Trim the tissue at the bottom edge or on all sides to eliminate empty resin

Microtomy

- Try cutting sections thinner, 0.4-0.5 microns, and stain with 1% toluidine blue stain.
- Try cutting sections thicker.
- Apply solvent vapors to sections still in the knife boat by waving a Q-tip or sliver of filter paper soaked with solvent over the sections. Solvents recommended were chloroform or ether (we have also used trichloroethylene). Note toxicity of these solvents by inhalation!
- Use a heat pen to achieve the same effect as above.
- Wet a plastic bottle cap with chloroform and hold the cap over the boat as the vapors evaporated. Chloroform vapor is heavier than air, and creates a downward force on the surface of water in your boat.

Section pick-up

- Pick sections up and float them on a container of hot water to stretch them before transferring them to the slide to dry them.
- Pick sections up with a loop (e.g., Perfect Loop) and immerse them in the water droplet rather than trying to roll the loop over to get the sections onto the slide. Come straight down on top of the water droplet with the loop (section side up) and let the sections float off of the loop and onto the water surface.
- Add ethylene glycol (maybe 1%?) or acetone (< 10%?) to the water drop on the slide (not in the trough). However, be aware

that section dimensions may change. One respondent also suggested adding ethanol at this step, but had not tried this with plastics.

- Switch to untreated glass slides. Superfrost Plus slides have a surface treatment that will make sections adhere more readily, not allowing them to spread out as they dry down and causing section puckering.

Drying

- Put a small open glass Petrie dish on the hot plate. Heat it. Put the slide, drip side down over the dish to evaporate. If necessary, flip dry slide over and heat to a higher temp to promote adhesion.
- Apply solvent vapors (chloroform, xylene) while drying the sections on the slide warmer. Several suggestions of how to do this were to place a drop of solvent in the lid of a petri dish over the sections while drying or to hover a chloroform soaked Q-tip over slide during drying
- Lower the temperature on the slide warmer so that sections will dry very slowly, then reheat after all water is gone to improve adhesion
- Raise the temperature on the slide warmer slightly then gently agitate the slide while it is on the hotplate to evenly dry the water.
- Place a few drops of acetone on a Q-tip on the frosted end of your slide, cover your slide with a Petri dish and allow the water to dry down on your hotplate. The increased pressure in the dome produced by the Petri dish from the evaporated acetone helps your sections to dry flat. Have the hotplate set hot enough to boil water.
- Heat a small open glass Petri dish on the hot plate. Put the slide, drip side down over the Petri dish during drying.
- Look at https://www.researchgate.net/publication/19412292_Flat_Adherent_Well-Contrasted_Semithin_Plastic_Sections_for_Light_Microscopy

Marie Cantino marie.cantino@uconn.edu **Mon Aug 15**

Immunocytochemistry:

immunogold negative staining of exosomes

We have a researcher who is interested in immunogold labeling and negative staining of exosomes isolated from mouse macrophages. So far we have done immunogold and negative staining with CD63 rabbit from SBI and didn't get any labeling. I would like to ask you, if you can recommend other companies we can get exosomal markers: CD63, CD9, etc. for immunogold negative staining on whole mount exosomes. **Maya Yankova** yankova@neuron.uchc.edu **Mon Aug 8**

The gold labeling will only work if the epitope is on the external side of the exosome membrane. Remember, they are like tiny balloons and only the external surface is exposed to the antibody. Not a solution, just something to consider. **Lee Cohen-Gould** lsgould@med.cornell.edu **Wed Aug 10**

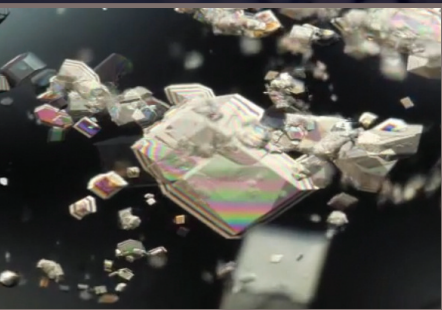
Although the remark is generally true, when it comes to CD it only makes sense if it labels external epitopes. For this very reason I would suggest to do pre-embedding labeling. Also you may consider a positive control to control if you are able to label the marker when you are sure that one is present. **Stephane Nizets** nizets2@yahoo.com **Thu Aug 11**

TEM:

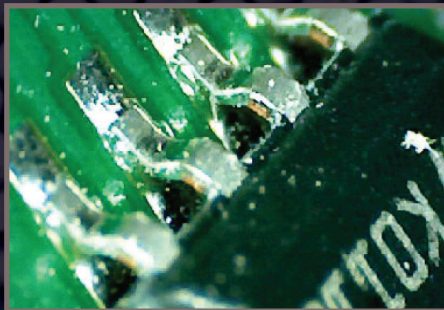
diffraction contrast

It looks like our venerable 30-year old JEOL 2000FX has come to the end of its useful life and we need to replace it. One thing this old machine

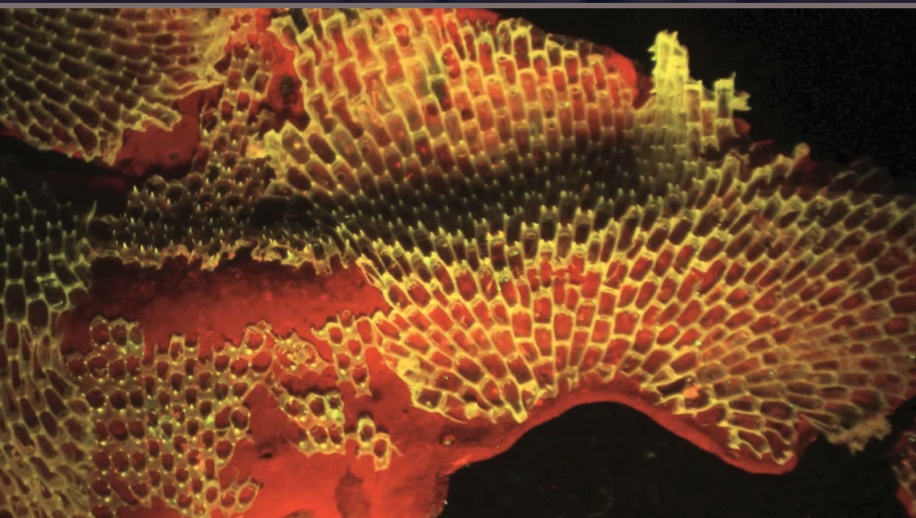
however you
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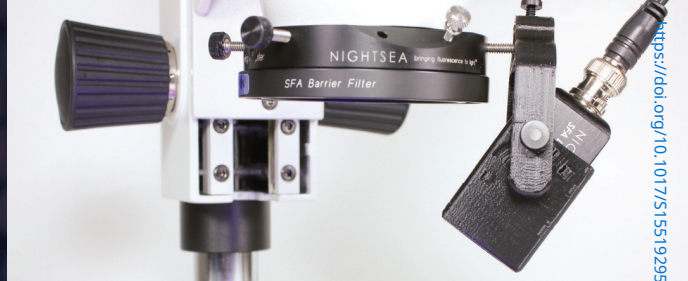
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could do, which I have not been able to do in any modern microscope, is take beautiful dark field images at low magnifications (<2000x), with perfectly parallel illumination and a large field of view. It seems that most modern microscopes don't even go down to such low magnifications and allow darkfield imaging; they just turn off the objective lens for 'low mag' mode. It is impossible to obtain low mag, diffraction contrast images on our 5-year old JEOL 2100 TEM; either the beam is too convergent, turning the diffraction condition into a stripe across the image, or the field of view is too small. The only solution I have found to date is to take lots of images and stick them together with Photoshop. Time consuming for 30+ images though! So my question is - does anyone make a TEM that can do this anymore? If so, which one? Can anyone send me a dark field image taken at 1500x on a modern TEM? **Richard Beanland** contact@integrityscientific.com Tue Jul 5

It's resolution! That's the spec that sells, and that is what they design and build for. Pole piece designs for higher resolution tends to have smaller gaps, and then to make up for field of view loss due to the smaller gaps, they have added "mini-lenses" before and after the objective lens (OL). On top of that, the OL aperture is not in the back focal plane, but in a hole drilled in the lower half of the OL pole piece. These new microscopes can't do decent dark field at any magnification, in my experience, but that is not what sells, so say adios to that capability. **John Mardinly** john.mardinly@asu.edu Wed Jul 6

TEM:

slow communication with Digital Micrograph

We have a JEOL 2100F TEM connected to a Gatan system running Digital Micrograph (DM; GMS 1.86). The computers are all 32 bit running Windows XP and we are not connected to a network. We converted the system to run at 80 kV for a few weeks and then brought it back up to 200 kV. When we did, the power supply on our GIF was dead and it needs repair. We currently have a loaner. I don't know when it blew and don't know whether it was related to the voltage change. The other thing that we noticed was that the microscope status was slow to update on DM. If we rebooted all of the computers, the status of mag would initially change in about a second. As you use the microscope, changing mag and modes, the update would get slower and slower until it takes several minutes for the parameters to be updated to the correct ones. All modes are slow, TEM, STEM, Diff, GIF, etc. This was not happening when we were at 80 kV, only after we were back up to 200 kV. The Gatan service engineer was not able to fix it, but he seems to remember something like this happening before. He's checking on it. I have a service call in to JEOL, and they seem to remember something like this happening on another system with an Omega filter. We don't have an Omega filter. Something is hogging the communication line between the two systems. Does anyone have a clue as to what might be happening? **Scott Walck** s.walck@comcast.net Tue Jul 5

I had a similar issue once on a (non-JEOL) SEM running Gatan DM, and it turned out to be a corrupted driver in the Gatan computer. Deleting and reinstalling the driver fixed the problem. **Phil Oshel** oshel1pe@cmich.edu Wed Jul 6

We faced a similar problem where the magnification update became very slow (etc takes about 10-50s to update) but we are using DM 2.xx version. What we did is to restart the VME from our TEM side. The VME button is at the back our JEOL 2100F TEM. So a routine is to shut down the JEOL PC, Gatan PC, restart VME and start the JEOL PC and subsequently the Gatan PC. **Tay Yee Yan** rongchigram79@yahoo.com.sg Mon Jul 11

This is just to add a comment about our microscope. It is also a JEOL JEM2100F (2012), running the JEOL TEMCON software

for control, with TVIPS camera and Software, and also SerialEM installed. No Gatan Software / hardware involved (except the Gatan 626 cryo-transfer holder). Occasionally, - once in a month or two - we face similar problems that on the JEOL PC in TEMCON, execution of "loading the alignment file" can be very, very slow (>> 30 sec or much longer), instead of 2 to 5 sec, as it should be. What we do is to restart the PC (not the VME) controlling the JEOL - usually, this is sufficient. Rarely, we also have to re-boot the VME of the JEOL. Then, the routine is to shut down the TVIPS PC, then the JEOL PC, restart VME and start the JEOL PC and subsequently the TVIPS PC. **Reinhard Rachel** reinhard.rachel@biologie.uni-regensburg.de Tue Jul 12

Is your computer networked? Is it a Windows system? I experience interruptions with my camera and other hardware drivers when certain automatic updates try to load on my confocal and other microscope computers. For a few years, I had regular "camera not found" messages on one of my research microscopes, and our computer people believe it was certain updates, which disconnect hardware while it installs. I blame Microsoft the most, but never tracked which vendor updates caused the problem. The problem occurs when the update is downloaded, but installation is not complete. Look for the yellow triangle at the lower right corner of your monitor on the start bar, and reboot whenever you see it. I've had scanning confocal imaging systems stop in the middle of a scan because the update just happened to come at that time. Your problem may be completely different, since it's an alignment file issue. My problems mostly went away when those equipment computers were taken off the network. **Gregg Sobocinski** greggs@umich.edu Wed Jul 13

EDS:

simulation with Desk-Top Spectrum Analyzer (DTSA)

I have a faculty member using DTSA to simulate EDS spectra collected with TEM EDS [using Sim Alien]. Sim samples are silicate minerals as "grain mounts" on a Cu grid [assumed config]. We are working our way through the Preferences set up for instrument, detector, sample. Using the general parameters of my TEM SDD detector [Thermo SDD] and using 100 & 200 kV acc voltages. Question: [ignoring the somewhat steep DTSA learning curve] are there specific issues or problems with using DTSA to simulate TEM EDS spectra? Assuming anyone out there does this kind of thing! Any sage advice would be appreciated. **Tom Williams** tomw@uidaho.edu Wed Aug 3

Nicholas Ritchie (the author of DTSA) maintains a forum here for questions on DTSA: <http://probesoftware.com/smf/index.php?board=32.0> **John J. Donovan** donovan@uoregon.edu Mon Aug 8

I have used DTSA for modeling S/TEM spectra using the MC software. I'm not sure I would use the software for high precision work, but it should be OK for things like detection limits, etc. There are 2 main differences between TEM and SEM modeling: beam voltage and the thin film sample. DTSA allows for a thin film sample where you can set the substrate to nothing. The kV should affect only the ionization cross-section. Once the overvoltage gets above 4-5, there is a relatively slow variation in the cross-section. (See for example, Williams & Carter 2d edition, Figure 4.4). Raynald Gauvin's work (M&M 2012 #1000) suggests that there should be little difference in the models at the higher kVs. The agreement of the models gives me a bit more confidence that the cross-sections are meaningful. You can always collect spectra from known samples and compare them to the MC models and see how close they come. If anyone has more detailed info about the modeling validity, I'd like to know too! **Henk Colijn** colijn.1@osu.edu Tue Aug 9

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