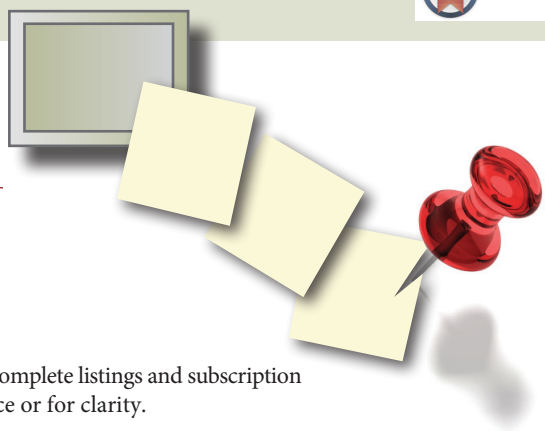

NetNotes

Edited by Thomas E. Phillips
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Selected postings from the Microscopy Listserver from March 1, 2017 to April 30, 2017. Obtain complete listings and subscription information at http://www.microscopy.com. Postings may have been edited to conserve space or for clarity.

Specimen Preparation:
silicon nitride or graphene support films

I'd like to make good support films for TEM. I used to be able to make films without holes in them but my tried-and-true method using 0.25% Formvar in ethylene dichloride hasn't been working (purchased solution or made in-house). Holey support films have become a way of life and one of the weakest links in my productivity. I would like to learn more about making silicon nitride or graphene films (they are so expensive to buy). Can anyone share a protocol? If necessary, I have access to a nice chemist and a clean room. ***Beth Richardson bethrichardson@uga.edu Fri Apr 28***

I cannot respond to the other films mentioned in your post, but as to Formvar, ethylene dichloride decomposes to release HCl, which impairs Formvar films. Be sure your reagent—or your Formvar supplier's—is fresh. In addition, it has been my experience that high-humidity environments make filmmaking problematic, and Georgia can be very humid this time of year. Bill Tivol wtivol@sbcglobal.net Sun Apr 30

Specimen Preparation:
formalin

I have a bottle of Formalin that has a white precipitate, probably polymerized formaldehyde from being stored in the refrigerator. Is there anything I can do to return the solution to normal? ***Jonathan Krupp jkrupp@deltacollege.edu Wed Apr 26***

Technically you can heat the solution to try and depolymerize the presumptive paraformaldehyde oligomers. I do not recommend trying this - it works occasionally, but hot formalin is almost never a good idea, even in a hood. Option 1: Send it to Hazardous Waste and buy another bottle. Option 2: Filter it and use it anyway. I have needed to use option 2 a few times over the years - mostly due to reagent shipping issues and/or "surprise!" samples. In my hands, filtered PF works okay for straight histology-level work, so-so (at best) for immuno work, and leads to a range of interesting results for EM samples. I would still recommend option 1—formalin is < $10/liter from Fisher. In a pinch, filtering for H&E or other optical microscopy will probably be okay. Aaron Barnes barnesa@umn.edu Wed Apr 26

It might depend: Is that bottle of formalin "labelled" (e.g., adhesive label indicating the producing company like Merck, Fisher or whatever company) and has high percentage of CH_2O or is it only a lower concentrated, "home-made" formalin solution made from concentrated formaldehyde solution? Has it been prepared with buffer or without buffer (= hydrous only) solution? If the former: usually if there has been included a "stabilizer" (besides 10% methanol usually calcium carbonate these days, in former days: "dolomite powder") the white precipitate originates possibly from the stabilizer. If the latter: it might be part of stabilizer (if the solution had not been filtered when it was mixed up) or it might be in fact deteriorating formaldenyde-oligo- polymers (as Aaron Barnes pointed out) or even precipitated phosphate or other ions (if the formaldehyde-solution was prepared with a buffer). In that case: If it were only one bottle (1 L or eventually also 1 gallon) I would do the following: Either filter or take out most of the solution by a pipet without disturbing the precipitate and use only for "normal" Histology as Aaron Barnes pointed out already. The problem arises here only that you do not know about the real concentration of the fixative. Or: dispose of (according to your legal and national safety concepts) after "neutralizing" (decomposing) the solution with, e.g., concentrated NaOH (which decomposes formaldehyde by means of the Canizarro reaction to produce formic acid (HCOOH) and methanol (CH_3OH). Since the blocking of free aldehyde groups (in fixation of tissues for immunocytochemistry) is done with glycine, sodium-borohydride or even ammonium chloride-solutions (or addition of these substances to formaldehyde solution) you could try to deactivate the formaldehyde solution prior to disposal by converting formaldehyde to alcohol(s). In addition, addition of bisulfite has been reported to be efficient. If you would like to look for other possibilities to dispose of old (perhaps deteriorated and of uncertain origin) formaldehyde solutions you might search by Googling for: | formaldehyde OR formalin AND deactiv* AND disposal | (also perhaps read my blog on formaldehyde-safe disposal on: https://www.researchgate. net/post/How_do_you_neutralize_ formaldehyde). Another possibility: Many aldehydes are respiratory irritants, and some, such as formaldehyde and acrolein, are quite toxic. There is sometimes merit in oxidation of aldehydes to the corresponding carboxylic acids, which are usually less toxic and less volatile. Procedure for permanganate oxidation of 0.1 mol of aldehyde $3RCHO + 2KMnO_4 \rightarrow 2RCO_2K + RCO_2H + 2MnO_2 + H_2O$. A mixture of 100 mL of water and 0.1 mol of aldehyde is stirred in a 1-L round-bottomed flask equipped with a thermometer, dropping funnel, stirrer, steam bath, and, if the aldehyde boils below 100°C, a condenser. Approximately 30 mL of a solution of 12.6 g (0.08 mol, 20% excess) of potassium permanganate in 250 mL of water is added over a period of 10 minutes. If the temperature rises above 45°C, the solution should be cooled. If this addition is not accompanied by a rise in temperature and loss of the purple permanganate color, the mixture is heated by the steam bath until a temperature is reached at which the color is discharged. The rest of the permanganate solution is added slowly at within 10°C of this temperature. The temperature is then raised to 70 to 80°C, and stirring continued for 1 hour or until the purple color has disappeared, whichever occurs first. The mixture is cooled to room temperature and acidified with 6 N sulfuric acid. (Caution: Do not add concentrated sulfuric acid to permanganate solution because explosive manganese oxide (Mn_2O_7) may precipitate.) Enough solid sodium hydrogen sulfite (at least 8.3 g, 0.08 mol) is added with stirring at 20 to 40°C to reduce all the manganese, as indicated by loss of purple color and dissolution of the solid manganese dioxide. The mixture is washed down the drain with a large volume of water. If the aldehyde contains a carbon-carbon double bond, as in the case of the highly toxic acrolein,

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4 mol (20% excess) of permanganate per mol of aldehyde is required to oxidize the alkene bond and the aldehyde group. Procedures for the laboratory scale treatment of surplus and waste chemicals (from an article launched to web by University of Geneva and really helpful): Formaldehyde is oxidized conveniently to formic acid and carbon dioxide by sodium hypochlorite. Thus 10 mL of formalin (37% formaldehyde) in 100 mL of water is stirred into 250 mL of hypochlorite laundry bleach (5.25% NaOC1) at room temperature and allowed to stand for 20 minutes before being flushed down the drain. This procedure is not recommended for other aliphatic aldehydes because it leads to chloro acids, which are more toxic and less biodegradable than corresponding unchlorinated acids. https://www.unige.ch/sciences/chiorg/matile/12-ProceduresforLabTreatmentofWasteChemicals.pdf Wolfgang Muss wij.muss@aon.at Thu Apr 27

Formalin is historically ~40% formaldehyde in water and laced with 5-10% methanol to inhibit polymerization. Thus, 10% "formalin" is 4% in formaldehyde. That is the historically recommended concentration of the gas as a fixative. 4% paraformaldehyde at 4°C (buffered or not) is also a very good treatment (10-20 min) for 'freezing,' muscle in thin tissues that are under various degrees of tension (e.g., in a partially to maximally distended urinary bladder), before they are immersed (or filled) with the same fixative. Fixation at 4°C is a very good fixative for both routine and special histology. I stopped using formalin in the mid 1970's, and substituted as follows. I usually make 20% HCHO from paraformaldehyde, and I can refrigerate it in 100 ml aliquots for over a decade without any polymerization. Formalin ought not to be used for anything in biological histology, unless the methanol is absent - which solution should not be called "formalin." Fred Monson fmonson@wcupa.edu Fri Apr 28

Microtomy:

certified thickness of sections

A customer of mine has an ISO certification of the EM lab coming. One of the problems is to reliably demonstrate the thickness of the 50 nm ultramicrotome slices and the semi-thick 500 nm slices in clinical workflow. Normally this is done in the field by using the interference color of the slices in the water tray which is not very accurate and depends also on the color spectra of the illumination system, the resin system, the accuracy of the ultramicrotome, the user, room situation; what shall I say more...? Did anyone of you had this problem and how did you solve it? This question is also posed to the manufacturers of ultramicrotomes, RMC and Leica. How do you handle this? Did anyone measure the thickness of the slices and how? Through-focus-series in TEM? Some setup in SEM? ***Stefan Diller stefan.diller@t-online.de Fri Apr 28***

Another thing to consider, is do you care about the thickness of the section (on water, grid, slide), or the thickness of the material removed from the block; i.e. the block advance (assuming the ultramicrotome is cutting all the material off after each advance). For our studies, the block advance, which can be related back to the original volume of tissue, is what we like to know. If you minimize compression (ultrasonic diamond knifes, different angles of knife and/or harder resin), then the block advance and the section thickness on grid begin to converge. Ben Micklem ben.micklem@pharm.ox.ac.uk Fri Apr 28

I thought the original method to determine section thickness accurately was to shadow sections, at a known angle with the sections on mica (or similar), and then calculate the height (thickness) of the section by the length of the shadowed metal deposit and the known angle. Admittedly, this is a lot of prep work and not something one would want to introduce into a "routine procedure." Lorenzo Menzel lmenz001@fiu.edu Fri Apr 28

Work has been done to determine section thickness in a more exact way than using interference colors alone. One method is to use the minimal folds method using the TEM (Small, 1968). Imagine crinkling a piece of paper so a fold forms in the middle in the *Z* axis, which will be twice the thickness of the paper. This fold can be measured on the TEM to get a relatively accurate thickness measurement. Ideally, measure multiple folds and get an average thickness. This method is addressed nicely in the following paper: When using samples with mitochondria, you can also use the cylindrical shape of those to estimate section thickness (Fiala and Harris, 2001). This is ideal if you have sections with very few wrinkles and was found to have an average thickness measurement very similar to that of the minimal folds method. Yet another method is to re-embed some sections you've cut and section them again at 90 degrees to measure the thickness (Bedi, 1987). Finally, laser confocal microscopes can be used to measure the section thickness with a reported accuracy of 1 nm (see Kubota et al. 2009). Connon Thomas connon.thomas@mpfi.org Fri Apr 28

Immunocytochemistry:

Immuno-gold labeled cellular structures on the membrane

We did immunogold labeling on MDA-MB-231 breast cancer cells grown on cover glass to see if the antigen would appear on the cell surface. The cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M cacodylate buffer pH 7.4. Immunogold labeling was performed before critical point drying and sputter-coating with Au/Pd. Images were taken on a Zeiss Sigma FE-SEM. Besides some scattered signals, a lot of gold particles are localized in one area. An example is here: https://goo.gl/photos/YPXZrwzNAukfsHBw7

Can you tell what the structure is? In addition, there were a lot of gold particles on the background/cover glass? Could you suggest ways of eliminating them? ***Zhuo Li zhuoli@coh.org Thu Mar 23***

This is not easy to answer in just a few words. To evaluate the reliability of any immunolabeling, it is required to at least check the negative controls: specimens incubated without primary antibody. Based on those results one can then look at background and what to do about it. If the negative control is clean, you are dealing with labelling based on binding of the primary. Pre-adsorption of the primary can help provide answers as to whether what you observe is specific or non-specific. If the negative control is not clean, the labelling is the result of interaction between gold conjugate and specimen. In that case, incubation protocols and specimen conditioning are the first thing to look at. Background, false positives can almost always be controlled. I will be happy to help with detailed suggestions if you would like, but since that might go into products and brands it would be better to do this off-list. Jan Leunissen leunissen@aurion.nl Thu Mar 23

Some thoughts about this problem: 1. It seems, at least, two possibilities for your picture. One is the structure (with gold particles) could be extracellular matrix, which is usually 'sticky' for gold particles, or could contain the antigen. Another possibility is that it is a broken membrane and you are viewing the inner membrane, which might have more antigen, or be sticky. You may want to do a 'control' with full fixation (glutaraldehyde + osmium), which provide you a better resolution of the structure. 2. Aldehyde group, especially that from glutaraldehyde is very 'sticky' to antibodies. Since you are not viewing the internal structure, I strongly recommend avoiding using glutaraldehyde, not even 0.1%. It may explain, at least in part, the heavy labeling on your cover glass. 3. Blocking with BSA, and/or serum could reduce the non-specific background. 4. I recommend the sample be coated with carbon, instead of Au/Pd. You can then confirm the gold particles with a backscatter electron detector (here is a reference I published many years ago - Localization of

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myosin on sperm-cell-associated membranes of tobacco (*Nicotiana tabacum* L.) - https://link.springer.com/article/10.1007/BF012790825. As Jan suggested, control is critical! Zhaojie Zhang zzhang@uwyo.edu Thu Mar 23

LM:

need 700-800 nm excitation for Zeiss AxioObserver

We need to excite in the 700 to 800 nm range and the Zeiss HPX-120 lamp we have is filtered to block wavelengths above 660 nm. Rather than customize the filter inside the lamp house, we are looking for an alternative light source for the 700-800 nm range with a liquid light guide or fiber that we could swap for the Zeiss HPX-120 on the days we need to image infra-red. Are there any simple light sources that fit this description that cost less than $3k? If not, how about ones that don't cost much more than this? ***Michael Cammer michael.cammer@med.nyu.edu Tue Apr 18***

I'd say it depends on how stable you want the line (frequency) to be. CD-ROM lasers are 780 nm, and super cheap thanks to economies of scale. Getting one coupled to the fiber of your choice would then be your challenge. Nathan McCorkle nmz787@gmail.com Tue Apr 18

HR-TEM:

good reference text

I'm struggling with Williams and Carter (2nd edition, I think) understanding HRTEM. I generally find their text approachable and easy to read, but not the HRTEM material, other than the math. Is there a text or article that is more detailed? Outside of the supposedly HRTEM sources that I have found have lengthy introductions to the basic, non-HR microscope, then brief descriptions of the math of HRTEM. What is a good read? I am a microscopist, with a B.S., so technical is okay, but I want a deep focus on HRTEM, theory and instrumentation. ***Kleo (Kathleen) Pullin kleopullin@email.arizona.edu Tue Apr 18***

Check out the Oxford text: *High Resolution Electron Microscopy* by Spence. A close second and less graduate-level math is found in *Transmission Electron Microscopy and Diffractory Materials* by Fultz and Howe (Springer). You are looking for a discussion of "Pendellosung," page 631, Fultz and Howe. To explain it to students at U of O that I taught, I built a coupled pendulum, where the one pendulum represents the non-diffracted beam and the other is the Bragg reflected beam. The coupling represents the lattice. The pendulum will stop and start, and that time can represent thickness of the sample. And so white spots in HRTEM, nodes, could be channels between atoms or atoms depending on how thick the sample is (how much time the pendulum swings). Pete Eschbach peter.eschbach@comcast.net Thu Apr 20

SEM:

LaB_6 emitters for EDS and WDS

A friend of mine is interested in buying a SEM with a LaB_6 emitter for imaging and, above all, analytical applications: EDS and WDS. However, he was told that LaB_6 emitters are not a good choice for analytical applications in a SEM, mainly due to stability issues, which—if I got it correctly—would require a long wait time before reliable spectra could be acquired. I have no experience with such a machine, so I cannot advise him about this. Maybe some of you can help me. ***Davide Cristofori dcristofori@unive.it Fri Mar 3***

I know of no problems with LaB_6 emitters with regard to stability, provided that the vacuum level in the electron gun is suitable for their use. Could you be confusing the instability problem with cold field emitter instruments, where their natural emission is prone to early and late instabilities during long operating sessions? Steve Chapman protrain@emcourses.com Sat Mar 4

LaB_6 emitters are unanimously considered stable, yet some of you pointed out that the filament has to be kept heated in order to assure a stable emission, even when not working, e.g. overnight. I would like to figure out how common this approach is, and what the life of LaB_6 emitters are which are operated in this way. It would also be very interesting and useful to know your experience about this point, if you want to share it. Davide Cristofori dcristofori@unive.it Fri Mar 10

Compare FEI 12T and FEI Quanta 400 ESEM, both tungsten emitters (exchangeable). Quanta 400 ESEM has average life = 60-80 hr. When Quanta vented to change specimen, entire column is vented with air (could be better with N_2, but not often). Tecnai 12T (120kV) has average life = 1000 hr. When you change specimen (single tilt), current condition of filament is maintained until vacuum in specimen chamber has recovered. Quanta filament is OFF overnight. Tecnai filament is under low current and minimum vacuum pressure 24/7 (10^{-8} with no break in vacuum in life). Fred Monson fmonson@wcupa.edu Fri Mar 10

LaB_6 emitters, like any other source, outgas when first heated and may then be unstable. A way of getting round this is to run the filament at a very low current, just to keep it warm, even if the instrument is not being used. I do not know if they do it now, but JEOL with LaB_6 systems set the filament heating at half value when you "turned it off". At very low emission there is no source evaporation, so the filament life does not suffer. Steve Chapman protrain@emcourses.com Fri Mar 10

To sum up the results of my little survey: it came out that keeping the LaB_6 filament heated, even not at a full range, is quite the standard approach for this emitters. Davide Cristofori dcristofori@unive.it Mon Mar 20

SEM:

Need help in understanding loss of image generation

Our Hitachi S-2700 SEM will no longer produce an image. We can only see a series of vertical lines of varying intensity with both secondary electron and backscatter detectors. We do have an electron beam - there are changes in intensity of the vertical lines during adjustment of filament current, beam tilt, beam horizon, changes in the lines if we move the specimen. We do lose the image if the HV is turned off on the secondary electron detector. A very similar pattern is seen with the backscatter detector, so it does not appear to be a detector issue. We have exchanged circuit boards and even the entire column (!) from a second identical scope used for parts. A commercial technician was unable to correct the problem. His best guess is that one or more of the lenses are not functioning. Any suggestions as to what the problem is? I can send images off-line. ***Jeffrey Thompson jthompso@csusb.edu Thu Mar 2***

Please feel free to send or post images to look at, but most likely, you have a simple deflection problem. Remember that in SEM lenses are only used to form the beam, but magnification (and formation of image) comes from ratio between area physically scanned by electron beam on surface of the sample and area of the CRT screen (or digital image) representing the image. If you see only vertical lines then chances are that primary electron beam is not scanned in Y direction over the sample, so in every line of image you are seeing information from the same exact line physically scanned by electron beam on the sample. Find a local tech who knows how to use oscilloscope and able to figure out amplifiers driving inductive loads (scan coils). Valery Ray vray@partbeamsystech.com Thu Mar 2

I agree with Valery, a deflection circuit is faulty. A deflection problem is also possible from the screen circuit. This can be check by

connecting the video output on an external monitor. This microscope has probably two stages of deflectors to scan the beam and each of them set with X and Y deflection coils. The most common problem is coming from power transistor on horizontal circuit. This component is more stressed because the scan speed horizontal is faster than the vertical. This electronic is sensitive to temperature of the room and of the cooling water. The faster the speed, the more those transistors are working. If you can set your microscope to slow scan mode when you do not use, it is safety for deflection circuit. High magnification position is also good. Nicolas Stephant nicolas.stephant@univ-nantes.fr Fri Mar 3

EDS:
zinc oxide and iron oxide reference standards

One of the users for our EM facility is looking for zinc oxide and iron oxide as standard reference for EDS analysis. If anyone can help. ***Ravi Thakkar ravi.thakkar369@gmail.com Mon Apr 17***

We may need more information before we can answer the question well—at least, I would need more. Is this EDS in conjunction with TEM or SEM? The form of the material would different for the two applications. If TEM, I do not have much to suggest since I do SEM. It seems they would want a thin film or powder. If SEM, they would probably want a homogenous, bulk sample. They can get bulk samples of iron oxide. Does it matter if it is Fe_2O_3 or Fe_3O_4? I am used to zinc oxide being a powder. It might be challenging to find it in bulk form. What do they ultimately want to know? Do they want to know if their material matches? Do they want to quantify the oxide? Do they want to see if they have excess oxygen? I often find users coming in with too narrow a question. When I found out the true issue, there is usually much more freedom in suggesting a solution. Why do they want the oxides? Most EDS systems will have standards built in for the elements. They are often quite good. I would not think that they need the oxides of the metals. What form is their sample in, bulk, powder, film? If it is not flat, polished, thick material, then accurate quant will be out of the question. I hope that the answer is not simply "Because". I find it much easier to help someone who is forthcoming with information rather than one who is dead-set on a single course of action. Warren Straszheim wesaia@iastate.edu Tue Apr 18

There are some sources for standards mentioned in this thread: http://probesoftware.com/smf/index.php?topic=889.msg5674#msg5674 John J. Donovan donovan@uoregon.edu Tue Apr 18

I am not affiliated with Ted Pella, but they do sell EDS/WDS mineral/oxide standards. http://www.tedpella.com/calibration_html/UHV-EL_Reference_Standards_for_EDS_WDS.htm Anonymous Tue Apr 18

EDS:
convert .spx data to .txt or .csv

I recently used an FEI Tecnai Osiris S/TEM to obtain EDS of my sample. I believe we have a Super-X EDX detection system in conjunction with Bruker Esprit software. The data was unfortunately saved as .spx. Is there any way I could convert it into .txt or .csv format? ***Anuja Bhalkikar anuja.bhalkikar@huskers.unl.edu Wed Apr 19***

Please look at the web page of NIST DTSA II software <http://www.cstl.nist.gov/div837/837.02/epq/dtsa2/>. It should be possible to import .spx file into this software and then export the data in .cvs format. Unfortunately I do not have any .spx files at hand, but I have tested it on .msa and .spc files, and it works well. Oldřich Benada benada@biomed.cas.cz Wed Apr 19

MT