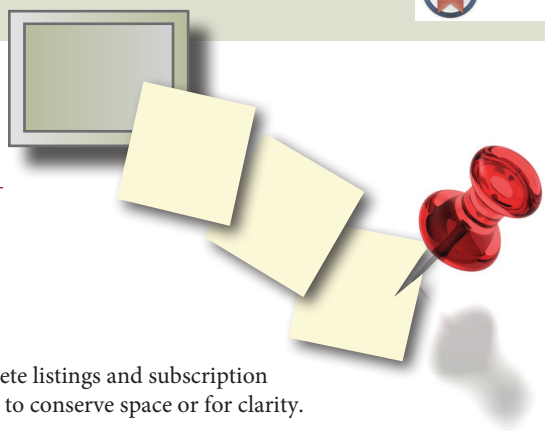

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NetNotes

Edited by Thomas E. Phillips
University of Missouri

phillipst@missouri.edu

Selected postings from the Microscopy Listserver from May 1, 2017 to June 30, 2017. 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:
gold labeling protein complex

I have a client who will be submitting outer surface proteins (OSPs) to resolve by TEM. He plans to use gold conjugated to the primary antibody, which will express an antigen on these OSPs. Does anyone have a protocol or know of any older articles related to this? Some concerns and questions I have are attachment of the OSPs onto a Formvar (or Parlodian) grid; using a routine immuno-electron microscopy procedure or a revised protocol; using a routine negative stain (uranyl acetate or phosphotungstic acid) or would low angle shadowing work more efficiently? ***Mary Ard maryard@uga.edu Wed Jun 21***

For uniform spreading of protein particles on the grid, it is best to have a glow-discharged carbon film on top of your Formvar or Parlodion. If that is not an option, bacitracin solution often helps, or even BSA. Then you can use any basic immunoEM protocol, which you can obtain from any major supplier or perhaps from community members here. Use minimum blocking first, to establish some binding, then you can include extra blocking steps or higher concentrations, if there is an objectionable background. This usually works quite well for a protein laying on a grid, with no obstacles, like when it is somewhere deep in the cell. What species is the primary antibody from? If possible, I always preferred to use Protein A-gold as a secondary probe. Much cleaner and more precise. It is best with rabbit polyclonals but will not work with most mouse monoclonals. Finally, the contrasting step - go with PTA (or ammonium molybdate) over UA: not as dark and spreads easier. Let me know if you need a sample protocol. Vlad Speransky vlad_speransky@tedpella.com Wed Jun 21

Specimen Preparation:
ethanolic phosphotungstic acid staining

I am trying to figure out what part of the cellular organelle or molecules the ethanolic phosphotungstic acid (E-PTA) binds to show the electron dense staining? I have found that it's commonly used for visualizing phospholipids or nerve ending. However, I do not know if E-PTA binds to lipids molecules or membranes. Does anyone know exactly what E-PTA binds to? ***Kyoung Jo kyoung.jo@rockets.utoledo.edu Mon Jun 5***

I guess you are referring to the ethanolic phosphotungstic acid (E-PTA) staining technique, which was introduced by Gray (1959). [cf: *Stains and Cytochemical Methods*, M.A. Hayat (ed.) 1993] "Ethanolic Phosphotungstic Acid," ch. 8, p. 284, where you can find a collection of stained and unstained material/tissue components, predominantly of/regarding nerve synapses. Perhaps you can find some or more of the information you are looking for in:

Hu et al., "Assembly of Proteins to Postsynaptic Densities after Transient Cerebral Ischemia," *J Neurosci* 18 (1998) 625–633.

FE Bloom and GK Aghajanian, "Cytochemistry of synapses: a selective staining method for electron microscopy," *Science* 154 (1966) 1575–1577.

FE Bloom and GK Aghajanian, "Fine structural and cytochemical analysis of staining of synaptic junctions with phosphotungstic acid," *J Ultrastruct Res* 22 (1968) 361–375.

Also, you can find hints on the nature of material that is stained (or less or not stained) by E-PTA in: Horowitz and Woodcock, "Alternative staining methods for Lowicryl sections," *J Histochem Cytochem* 40 (1992) 123.

Perhaps further reading: Cattini & Davies, "Observations on the kinetics of uranyl acetate and phosphotungstic acid staining of chromatin in thin sections for electron microscopy," *Stain Technology* 59 (1984) 291–304.

Gluenz et al., "Structural asymmetry and discrete nucleic acid subdomains" in the *Trypanosoma brucei* kinetoplast," *Mol Microbiol* 64 (2007) 1529–1539. Wolfgang Muss wij.muss@aon.at Tue Jun 6

LM:
unknown microbe

I was looking at some pond water in a light microscope, and managed to capture this guy. Being a physicist, I have absolutely no idea what it might be, but I am sure that someone on this list will know what it is. Here is a video that I took of it: http://www.jkraft.net/unknown-microbe.mp4. *Also, if anyone knows of a good internet source of information on how to identify the random little slimy bits in pond water, I would appreciate it.* ***Justin Kraft kraftpiano@gmail.com Thu May 4***

Contact the folks in Seth Tyler's lab at University of Maine – they run a "global worming" website about metazoan microfauna, but they are mostly marine: http://globalworming.umaine-biology.net/. Pennak's "Freshwater Invertebrates of North America" is a good reference. After that, you need to get into the literature for each group, or google the group name, then click "images". But. Squished by the coverslip and without a scale, it's hard to tell. The squishedness prevents the critter from exhibiting its true form. There are several sets of somethings … 2 near the anterior end, just behind the mouth (on the right), one about ½ way along the body, on top of the animal and hard to see (about where the gut makes the sharp bend), and another 2 spaced equally behind this, on the "top" of the animal as it sits. This makes me think it's a seriously uncomfortable tardigrade. Too flattened by the coverslip to really show its true form. Common animals, but you need to put feet on your coverslips to give critters like this some room. Great dark-field subjects as well as DIC. Through in a green filter below your condenser, and use the 40× DIC annulus with the 10× objective, and you'll have dark-field (100× annulus and 20/40× objective will work on some microscopes). Given how poorly studied these groups are, it could be a new species. Philip Oshel oshel1pe@cmich.edu Fri May 5

Looks like it may be a freshwater Oligochaete, a type of Annelid worm (think earthworms). Couldn't tell you what species it is though; that would take some careful keying as there are hundreds of them in North America (if that's where you are!). If you look closely, it has what are called chaetae (bristles or hairs) in bunches on its body. There are quite a few keys to freshwater creatures. Here are just a few: Stroud Water Research

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Center Identification Guide to Freshwater Macroinvertebrates (a nice, very basic visual guide to more common groups): http://www.stroudcenter.org/education/MacroKey_Complete.pdf. Guide to the Freshwater Aquatic Microdrile Oligochaetes of North America (more specific to these worms): http://www.dfo-mpo.gc.ca/Library/33909.pdf. Guide to freshwater microorganisms: https://www.msnucleus.org/watersheds/mission/plankton.pdf Freshwater Macroinvertebrates of Northeastern North America (a book I used in my undergrad to identify slimy bits in pond water) https://www.amazon.com/Freshwater-Macroinvertebrates-Northeastern-North-America/dp/0801496888/ref=pd_lpo_sbs_14_t_1?_encoding=UTF8&psc=1&refRID=GE75176NY822DJV8BM3Y. As was previously stated, it may be helpful to reach out to a freshwater invertebrate lab if you are still curious. Connon Thomas connon.thomas@mpfi.org Fri May 5

LM:

HBO 100 light leak

I am searching on literature about the potential hazards of having light leaking out of an HBO 100 illuminator. There is plenty about how dangerous a mercury discharge can be if a lamp explodes. Can someone direct me or share with me information concerning the dangers of users being exposed to light leaking from a lamp housing? ***Mike mikegf@hmamail.com Mon May 8***

We used HBO 100 lamps in out of our Reichert MeF3 metallograph for several decades. Reflected light out of the illuminator cooling vents or leaking light at the illuminator/microscope joint was never cautioned against; this was 30+ years ago, but I suppose the explanation from the factory trainer was that the internal surfaces were sufficiently poor optical and UV reflectors that once the light was reflected it wasn't a concern. Of course, the potential for permanent retina damage made direct viewing of the lamp an absolute prohibition - never ignite the bulb without the protective cover in place or look directly into the optical path. That's somewhat obvious to us, but the general public doesn't comprehend the extreme brightness of these lamps. Rick Ross richard.ross@allisontransmission.com Mon May 8

TEM:

LaB_6 filaments/ cathodes

Has anyone experienced problems with LaB_6 cathodes in their TEM e.g. high dark current, beam instability? I have a vague recollection of historic issues mentioned on the List server. The two LaB_6 that we purchased in 2014 and recently installed in our CM10 FEI TEM are extremely problematic. The first one caused an array of issues notably high dark current, beam instability flashing/discharging. Much trouble-shooting searching was done by lab staff and FEI service engineers—cleaning of anode, Wehnelt and insulator, and replenishing gun-insulating oil to rule out other potential causes. We also tried the spare LaB_6 cathode, which demonstrated the same issues. Installing a tungsten as a last resort resulted in the EM quickly returning back to usable status with none of these issues. I have used LaB_6 from this manufacturer for years without any problems. Perhaps it is time to move on to another. I would be interested to hear from others experiencing these or similar issues. ***Levina Dear levina.dear@health.nsw.gov.au Mon May 8***

LaB_6 units are extremely dependent on vacuum level for their stability. Looking at the tests you have run I would be interested in the vacuum in the gun, not as given by the microscope but by monitoring as near as possible to the gun area, perhaps moving a microscope gauge nearer to that area if possible? Steve Chapman protrain@emcourses.com Tue May 9

Thank you for all the advice regarding LaB_6 filaments. Summary of some of the responses: (i) Cleaned the legs of the filament (emery paper then washed with acetone) resolved the problems. (ii) LaB_6 filaments have a limited shelf life. (I have not seen a use by date on any that we have purchased). (iii) Extremely dependent on vacuum level for their stability. (iv) LaB_6 filaments, from crystals cleaving to bases coming loose - and it seems from multiple suppliers. Result: A new LaB_6 was purchased from the same manufacturer; it was installed and is working beautifully, so good to see such a bright green glow again - problem fixed! I am waiting to hear back from the manufacturer regarding the two LaB_6 that were returned. Levina Dear levina.dear@health.nsw.gov.au

TEM:

introducing FOCUS: The interface between data collection and data

We would like to draw your attention to a new software system called FOCUS, which we find quite handy to work together with automated cryo-EM data collection, as done for example with SerialEM. FOCUS is a C++ / Qt front end, running own and third-party software in the background. It features a GUI that allows you to define and edit parameters and C-shell or Python scripts, which are then executed in queues. Users can add or edit their own scripts, to adapt FOCUS to specific workflows or tasks. FOCUS comes with a set of default scripts, ready to run. Installation of FOCUS requires also the installation of any third-party programs that you want FOCUS to utilize, such as IMOD, EMAN, FREALIGN, or MotionCor2, UNBLUR, Zorro, CTFFIND4, gCTF, gAutomatch, or others. If you install FOCUS on a strong Linux machine on a computer adjacent to the automated SerialEM run, then FOCUS can for example be used to: monitor the file system of the SerialEM run fetch newly recorded movies from the SerialEM computer, together with the pixel-defect-list and gain reference files, unpack the compressed movies (TIFF ZLW), gain correct, Fourier crop 8k to 4k, or to any pixel size you want (optionally) drift-correct (with ZORRO, MotionCor2, or Unblur) computer 2D averages of the movie, and FFTs of all files measure defocus (gCTF or CTFFIND4) pick particles (gAutomatch) present the last recorded image and its FFT (before and after drift correction), and the drift trajectory plot and CTF Thon ring plot on a website that you would have to setup (ours is at https://status.c-cina.unibas.ch. Ours is open and shows blurred images, but you can protect it with a password and then display the crisp images) present the statistics of each recorded image on that web site (as plots over time) organize all recorded movies in a large table (similar to an Excel sheet), which can be sorted by various parameters such as grey value (which images are too dark (contaminated, beam lost) or too white (empty hole?) defocus (which images are in focus or totally out of focus?) CTF resolution (for which images is CTF determination difficult?) iciness (which images have a too high ratio of crystalline ice, or show devitrified sample?) drift (which images have too long or too short or too jittery drift trajectories? which images have too high remaining interframe drift?) and various other parameters. This table can be sorted by any of such parameters, and images can be moved into a TRASH folder with one click. Remaining images can be exported in folder structures and STAR files ready for subsequent processing in, e.g., Relion. FOCUS can also be used for tomography sessions, to: recognize parameters from the file name (e.g., specimen number and tilt angle) and organize files accordingly drift-correct each recorded movie at a certain tilt angle, while taking the current electron dose and the prior electron dose from earlier tilt angle recordings on the same specimen into account for electron-dose-dependent B-factor resolution filtering compute one 2D image per tilt angle movie re-organize the tilt angle images by tilt angle (-60,-59,…,0,…,59,60) when recorded in the Hagen scheme (0,1,-1,-2,2,3,-3,-4,4,5,…60,-60) as above: monitor data collection progress on a web site, sort data, assist in manually pruning of data, export data in MRC stacks with one frame per tilt angle. Write-out

metadata for the stacks into STAR files FOCUS can also be used for 2D crystal sessions, where it can do the entire 2D crystal processing automatically (the full functionality of the 2dx software package is included in FOCUS). This allows direct 3D reconstructions during the 2D crystal data collection session. FOCUS maintains a batch queue, with which is runs all processes above in parallel. We have installed this on an Ubuntu PC with 2 ×12 cores (= 48 threads) 256 GB RAM (of which 96GB are used as a RAM disk. More would make life easier.) 70 TB HD (RAID5) 2 × GTX1080 GPU We also use a 40" 4k monitor, which is great to display the images full-screen in full quality. We have one such system per automated cryo-EM microscope, which is able to keep up with single particle data collection of 40-frame 8k movies at a rate of 90 movies per hour (Fourier cropping, drift correction with MotionCor2 on 5 × 5 patches, CTFFIND4, gautomatch, etc.), and is thereby on par with data collection. FOCUS is described in Biyani et al., *J Struct Biol*, 2017 (https://www.ncbi.nlm.nih.gov/pubmed/28344036). It is also available on bioRxiv at https://doi.org/10.1101/105452. The software is open-source and freely available in precompiled versions for Linux and OS X. A manual in wiki form is available, all at http://focus-em.org. Nikhil Biyani, Ricardo D. Righetto, Robert McLeod, Daniel Caujolle-Bert, Daniel Castano-Diez, Kenneth N. Goldie, and Henning Stahlberg. Henning Stahlberg henning.stahlberg@unibas.ch Wed May 10

TEM:
repair recommendations

We have a Zeiss CEM 902 Transmission Electron Microscope that is currently displaying the following error code: 3-1: Output voltage of high-voltage measuring tube below 0.1V (broken measuring line, defective measuring instrument or contaminated measuring tube does not ignite). Zeiss no longer supports this instrument so we are looking for recommendations for maintenance and repair. Any suggestions? ***Amy Replogle areplogle@pugetsound.edu Fri May 12***

When does the error code light up? Only after quite a while when the EM was running? I heard of a problem that in rare cases the vacuum becomes too good (e.g., better than 10^{-7}) and thus the voltage exceeds the minimal value which is necessary. The people helped themselves via a needle valve continuously admitting a smallest amount of air. Peter Heimann peter.heimann@uni-bielefeld.de Sat May 13

First, it would be useful to know if the error comes from a Pirani or a Penning tube. Can you be more specific? Can you relate the error code with the location / kind of the measuring tube? If Penning, I would first try to clean it. If the Penning is too contaminated, it looks like the vacuum is ultra-good. Procedure would be to set the part of the vacuum system the tube is fixed at on air, then switch off the microscope completely, since there might be high voltage on the tube. You can send me an image of the measuring tube(s) and I can check if I have a working one to send over. Stefan Diller diller@stefan-diller.com Sat May 13

TEM:
question regarding K-kit

Has anybody here used K-kit to analyze liquid samples under TEM? Is the K-kit useful for liposome too? What kind of specimen holder is needed? Will the FEI double tilt holder work? ***Ravi Thakkar ravithakkar@vet.k-state.edu Fri May 19***

We have worked with the K-kit system in our facility as well as working with others who are using the K-kit for a number of liquid systems. In general, if a K-kit loaded with organic substance that without staining, the imaging results mostly would suffer low contrast under TEM. The liposome is just one of the examples. A liposome has an aqueous solution core surrounded by a thin hydrophobic membrane, in the form of a lipid bilayer. Due to the contrast issue, the TEM images for some smaller sizes of liposomes are easily looked blurry, and poor for being clearly identified. Per our actual test results, we suggest that Cryo-TEM can be a better option for those smaller liposomes. However, one also can use K-kits for the observations of some larger liposomes under TEM. Since K-kit is mounted on a standard copper grid for TEM observation, it's surely compatible with most kinds of TEM Holder. Will FEI Double tilt holder works? Some kinds of FEI double tilt holders require using a small screwed cover ring to lock the copper grid fixed at the holder's front stage. It usually needs to use a dedicated tool which with a small hexagonal head to fasten them together. In this case, due to K-kit body on the copper grid is too thick, it will cause the tool to fail when screwing down the cover ring. Gene Rodek erodek@2spi.com Mon May 22

SEM:
air-sensitive sample

I have a user with a sample that must be kept under an Argon atmosphere until placed in the SEM exchange chamber, and I have never had to deal with this situation before. I am wondering if the following procedure will work, or if there are any issues with it. Our Hitachi S4700 SEM has a separate exchange chamber prior to the Sample chamber. We propose to tape a flexible glove bag around the frame of the SEC, containing the samples and mounts. We would first evacuate the bag using the rotary pumps and then seal the exchange chamber. Then, we will flush the glove bag several times with argon using the glove bag ports only. Once the argon atmosphere is present, the samples will be removed from their carrier, placed on the SEM mount in the glove bag, then into the exchange chamber, and evacuated. Once vacuum is reached, the sample will be put into the sample chamber. I hope that all the argon will be gone by then. After viewing, there is no current plan to try to keep

the samples from the atmosphere. In addition, over 90 researchers from several faculties use our SEM. Would a temporary setup like this with the argon cause any problems or concerns down the road? As I said, this is the first time for me trying something like this, and I do not want any surprises. ***Pat Scallion pscallio@dal.ca Tue May 30***

Your concept sounds feasible but I'll let the experts on here comment further. I thought you might want to have a look at 2 other solutions that I have seen: 1) VacuShut sample transfer device - this was sold by Agar Scientific but I don't see it on their website now. It was invented at Karlsruhe Inst for Tech. Here is a flyer PDF showing how it works. Maybe the inventors listed on this know where you can acquire it now: http://www.int.kit.edu/downloads/INT_Research/Flyervacushut.pdf. Also, Transfer Module from Kammrath Weiss - a more elegant solution (and probably more expensive): https://www.kammrath-weiss.com/en/products/materials/transfer-module.html Mike Toalson mtoalson@nanoimages.com Tue May 30

It sounds like something I did back in 2002 on a similar SEM back at the Electron Microscopy Center at Argonne National Lab. Should work just fine and an inert gas like argon will not cause any problems. Science and research are all about having fun and trying new things. Roseann Csencsits roseann.csencsits@schafercorp.com Wed May 31

FIB:

typical metallic purity for FIB source

I will have to replace my LMIS soon and was just wondering what they start as. Seems like the less pure, the less ideal your focused beam spot would become, and more 'chromatic' aberration probably. Just looking around I find 99.99% pure through 99.99995% available. So where would these be relative to what precision FIB would commonly use? I would guess that for some applications, a custom ion mill could tolerate a wide range of purity (not that I can imagine one at the moment). It leads to the question of, do all impurities ionize, or would some remain as 'slag' that prevents transport (flow) of clean metal to the emission point. ***Nathan nmz787@gmail.com Thu Jun 1***

When I was at Drexel, I remember the FIB scientist discovered (with the help of another scientist at Applied Beams) a solution as to why all of our ion beam images and milling patterns had these ghostly shadows present. For example, if you tried to drill a very small hole into a silicon nitride membrane, instead of single hole you would get two! This turned out to be because our ion source had two different isotopes of gallium present, and each isotope experienced a slightly different path through the optics and was focused to a crossover that was slightly laterally displaced with respect to each other. The purity of the source is certainly one thing to consider but apparently the isotopic distribution of even a pure gallium source is another. This doesn't answer your question directly but I thought you might find it interesting. I imagine the FIB source suppliers go to some extra lengths to make sure that the ion source is isotopically pure Ga-69. Chris Winkler microwink@gmail.com Thu Jun 1

EDS:

parameter definition

We just got an up-date on our IXRF EDS system. One parameter we get from the semi-quant routine is MDL (3sig). We are not sure we know what that means. It has been suggested that is an actual lower detectable limit for that element, while others suggest it is a parameter defining detectable signal over background. Thoughts anyone? ***Frank Karl frank_karl@ardl.com Sun May 7***

Not having seen any replies so far, I will jump in. I had an IXRF system for over ten years; indeed, we were one of the early customers. However, I cannot recall the specifics of their MDL numbers. MDL should mean the actual lower detectable concentration given the parameters of the collection. Therefore, it should drop with additional counts because the relative noise in the background is being reduced. If it does not change over time, then it could be a defined parameter which would not be correct in my opinion. You may also want to look into the practicalities of their system and its calculations. (Remember, my information is several years out of date.) An old version of their software fitted the background and subtracted it and then rectified the data. That is, all of the negative counts in the spectrum were set to zero. That resulted in a positive integral and result for virtually any element even when none was present. The reported amount diminished over time (approached zero) for those cases where the element was not present. It may have been that the reported level was less than the reported MDL. I should have compared the number to the MDL and ignored results less than MDL. Many systems flag results that are less than statistically significant, but some do it more clearly than others do. However, I think it would have been better if they had let the result wander around zero (both positive and negative) and settled in closer and closer to zero with time. I have heard from various software people that negative results are incomprehensible for many users so they (the developers) take steps which end up biasing the results. For the record, I reported this matter to IXRF on at least two occasions and it had not been resolved the last time I checked which was several years back. I was told it was going to be addressed. I certainly hope it has been fixed by now, but we may all be able to cite software issues that languish on the do-do list. Maybe one of their engineers is following the list and can clarify the matter. Warren Straszheim wesaia@iastate.edu Wed May 10

MT