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NetNotes

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Specimen Preparation:
site-specific protein labeling

Could anyone suggest what would the best feasible way to label my membrane bound protein of interest in cell (facing toward the cytosol) to study its orientation and/or interaction with a membrane using single molecule techniques like FRET/ FCCS/FLIM-FRET, etc. I am particularly interested in studying the orientation of a specific domain of the protein with respect to the membrane in the live cell. From literature, I have learnt of applying a specific ligand (bound to a fluorophore) that would bind to the active site; but that might not work for my case as the protein domain is surface exposed and we don't know of any such ligands binding to that surface. Also, is there any hope of using a small peptide directed to the protein domain (or, the amino acid stretch) bound to fluorophore for this purpose? Any help will be highly appreciated. ***Suparna csuparna1@gmail.com Mon Oct 17***

You are putting the bar very high. Considering cell membranes are dynamic and EM techniques require cells to be killed, don't expect it to be an easy task. I would say the first consideration is the distance between your protein domain of interest and the cell membrane. There is a chance that this distance is too short to be resolvable by classical fluorescence microscopy techniques. Thanks God Stefan Hell came on earth to save fluorescence microscopists and now we have fluorescence microscopes with very high resolution, however there is not always one nearby. You are talking about FRET, meaning you probably expect distances of several nm. In this case, I don't see which technique would allow you to resolve this distance in live cells. Sure you'll see a fluorescence due to FRET but you won't be able to locate it with respect to the membrane, I am afraid. Please also consider the optical thickness of the plane observed in LM. I am not really up-to-date in confocal microscopy but I think you can at best get optical sections of 100nm, so you are limited in resolution in the *Z* axis as well. If distance is not such a big challenge than you can perhaps consider expressing your protein/domain with a fluorescent tag, like GFP or RFP? Stephane Nizets nizets2@yahoo.com Mon Oct 17

Specimen Preparation:
LR White

How is LR White supplied and used? I am getting confused by the labels that say "catalyzed" and when to use the separate "accelerator." Is the benzoyl peroxide accelerator only added for doing cold cures? What is the catalyst that is already mixed in? Does this affect the shelf life or heat cure? I thought I had this figured out but this morning a student brought me a vial of LR White that had polymerized overnight in the refrigerator. No accelerator added, straight from the stock bottle. Can't figure that one out. I am now confused about this stuff and need some remedial education. ***Jonathan Krupp jkrupp@deltacollege.edu Fri Oct 21***

LR White, (London Resin White) is used mainly for post-embed immuno-electron microscopy. The cut surface allows for epitopes to be exposed and labeled with antibodies without the need to etch or antigen retrieval techniques. Most times it contains the catalyst so polymerization can be done at 50°C. Check with the manufacturer but it can also be UV polymerized (you may need to add a different catalyst). Most samples are lightly fixed (no high concentrations of glutaraldehyde) not osmicated and dehydrated up to 70% ETOH (I usually go to 90% as the 70% is sometimes difficult to completely mix with the resin). Uranyl acetate and small amounts of tannic acid may be used in lieu of osmium. Sections are collected on Formvar grids because the resin is beam-unstable. Semi-thin sections can also be generated to try immunofluorescence experiments. Michael Delannoy mdelann1@jhmi.edu Mon Oct 24

TEM:
focusing Zeiss EM109

Our facility has a secondhand Zeiss EM109 TEM, which we've recently had serviced. Part of this servicing included getting the coils adjusted mechanically, and it should be in peak condition. However, I have a lot of difficulty getting things into clear focus. I've gotten (I think) quite experienced with aligning and stigmating it, but that doesn't seem to fix things — indeed, after capturing a blurry image and realigning, the next image of the same sample often looks worse. I suspect that the stigmation is off, but I'm not sure; often I can get better results by systematically capturing images at different focus settings (though of course even with a digital camera this takes a long time, and doesn't do the specimen any favors). Are there any tricks to this? One question that I have in particular concerns the projector stigmation. I set this using the caustic, which I try to get to look like a Mercedes-Benz symbol (a perfectly symmetrical three-pointed star in a circle). However, changing the low-mag focus (the taller inner knob that doesn't click) changes the appearance of the caustic, such that at one focus setting (say, with the caustic spread out) the caustic is symmetrical, and at another (in this case when it's smaller) it isn't. Is there a "sweet spot" that I should have that focus set to (i.e., should I make the caustic as small as possible while still visible, or as large)? I should note that I have experimented a little with the stigmation while viewing specimens, and haven't noticed any effect (unlike my experience with a Tecnai-12, on which I could correct the astigmatism just looking at the image). But I do suspect there is a stigmation problem somewhere in the scope, and just need to know how to fix it. Any advice on the care and feeding of this microscope would be much appreciated. ***Aaron A. Heiss aheiss@amnh.org Wed Sep 14***

It is very many years since I used a Zeiss 109, so my memory of its layout is long gone, but I may be able to help you? The image in any TEM is created "in focus" by interaction between the objective

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lens and the lens which immediately follows it. Any problem with the clarity of this image will be related to the objective lens setting and the setting of the objective lens stigmators. Problems in any other lens (other than instabilities) will not have any influence on the image sharpness. You say that the instrument has been serviced by a person who knows the 109, so we must expect it to be free of high voltage or lens problems. That brings us back to the objective lens, but most of all in your case, the objective astigmatism. You note that with a through focal series the image improves, this goes hand in hand with astigmatism. At true focus the astigmatism will be minimized but as you move out of focus it will destroy the image quality. Please look into the objective stigmator, it may be a double strength system like all modern instruments, or it may be in the style of early instruments. Early instruments used a stigmator, which provided a device to change the stigmator field strength, combined with the ability to change the direction of that field. This system was called strength and rotate, and it may be the style on the 109. From the point of best focus change the strength control until you see it change the image. Then change the rotate control until you find the point that improves the image quality. Then back off the strength slightly, check the rotation, check the focus. Repeat this procedure until the image is satisfactory. Steve Chapman protrain@emcourses.com Thu Sep 15

TEM:
B field strength in JEOL 2100FX

Do you know someone who might have measured it or have a value that is at least by 10% close to the real value? ***Hasan Ali hasan.ali@angstrom.uu.se Thu Sep 22***

See Christenson, K., & J. Eades J (1986). On "parallel" illumination in the transmission electron microscope. *Ultramicroscopy*, 19(2): 191–194; http://doi.org/10.1016/j.ultramic.2006.04.029. You could do the experiment described in this paper to measure the Larmor frequency. With near-parallel illumination, take a series of images over a large Z range, then align the images in e.g., MIDAS which is part of IMOD software (http://bio3d.colorado.edu/imod/), this will give the image rotation per focus step used, the Larmor frequency. Once you have the Larmor frequency, you can calculate the field strength in the immersion lens. This paper from the same authors has a bit more detail: K Christenson, and J Eades, "Skew Thoughts on Parallelism," *Ultramicroscopy*, 26 (1988) 113–132. Wim Hagen wim.hagen@me.com Thu Sep 22

SEM:
staining Cu

We are using an older model SEM to image samples with copper structures on the surface and just a few nm below the surface at the same time (typically buried under a thin layer of glass). Since the copper layer below the surface is just covered by this very thin glass layer, even with lower acceleration voltages it is very hard to distinguish on the images whether the imaged copper structures are located on the surface or below it. As our goal would be that the copper on the surface clearly looks different from the copper buried under the thin glass layer, we were wondering whether we could use a staining procedure. For instance, we are aware of a wet chemical procedure allowing to tin-plate copper. However, without trying it we don't know if it would result in a considerable different contrast in the SEM as the atomic mass of Sn is very close to the one of Cu. Wet chemical gold plating on the other hand involves not only expensive but also highly toxic chemicals. Are you aware of (wet chemical) staining procedures for copper that produce a considerable contrast and are preferably inexpensive as well as not highly toxic? Is there maybe another technique we could use? ***Stefan Schoenleitner dev.c0debabe@gmail.com Wed Sep 7***

There are few things you can try to distinguish between exposed and slightly buried Cu conductors: 1. Based on your mentioning of "atomic mass of Sn is very close to the one of Cu" I am suspecting you may be using backscattered electrons for imaging - try secondary electron detector imaging at lowest acceleration you can afford without losing too much resolution. 2. Apply negative bias to the sample (retarding field) to reduce landing energy of primary electrons. This could work or not, depending on geometry of your instrument, stage, and your instrumentation skills/capabilities. 3. Electrolysis nickel-plating chemicals cost less than Au plating kits, though either one is safe enough to do in ordinary fume hood with gloves and common sense. 4. Exposure to iodine vapor in humid atmosphere (room air) would corrode exposed Cu fairly quickly, making it look "fluffy" or "rough," while buried conductors will remain intact for a while. The tricky part would be to image/scan the sample fairly quickly - once started the corrosion would eventually consume all the Cu layers. You want to image the exposed layer once it has been just slightly corroded on the surface, and immediately polish it away to prevent damage for the underlying layers. 5. Either sulfuric or nitric acid with H2O2 should etch Cu crystallographically, making the exposed surface look different than metal protected by dielectric. You would want to experiment with "disposable" devices and develop reliable procedures prior to immersing the "real" one. The same is true for plating, by the way. 6. Image the chip, etch away Cu with FeCl3, wash-rinse, and the image same area again. Buried under dielectric Cu would be on both images, while exposed would disappear after etching. You would need to develop procedures and control time/temperature/concentrations of etchants to get repeatable results. 7. Do forward-scattered imaging, or even regular SE imaging at glancing angle - it would be much more sensitive to the presence of a thin dielectric layer. Foreshortening correction would be required. Valery Ray vray@partbeamsystech.com Sun Sep 11

SEM:
remote imaging

We have previously instituted remote imaging on our FEI Quanta 200 for broadcasting live demos to our local school area. We also wanted to do the same with our Hitachi S4700. Although we can load the server/client software (TightVNC) and get a connection, the image does not port to the client pc. We get a "purple screen". Has anyone been able to get the live (or captured) image from the Hitachi onto a network client? ***Wallace Ambrose wambrose@unc.edu Wed Oct 12***

I believe you're seeing the effects of hardware-acceleration with the video. What I am guessing is happening is the microscope image is transferred via DMA (direct memory access) to the video card directly, bypassing the operating system. VNC however grabs the video frame buffer from the operating system, which doesn't have any information (because after that image is transported to the video card, the DMA transaction fills in the purple section before being sent out to the monitor). What I did for an older 1990s FEI FIB 200, was to use a VGA video cable splitter: http://www.siig.com/av-products/splitters-distribution-amplifiers/vga/1x2-compact-vga-splitter.html and then a VGA2USB frame grabber: https://www.epiphan.com/products/vga2usb/ Then get another computer, an old desktop or laptop or even a new mini-PC like an Intel NUC or compute-stick or RasperryPi (<=$40), which can stream/save the video however you like. You'll just need to get a splitter and grabber for your specific video cable (I'm guessing VGA or DVI): https://www.epiphan.com/

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products/#usb-video-grabbers Nathan McCorkle nmz787@gmail.com Thu Oct 13

XEDS:
problem with peak splitting

We have a problem with an older Noran Si(Li) detector running on a Voyager platform (it is about 19 years old!) that I have not come across before. Suddenly this week instead of getting a single peak, all of the peaks in the spectrum are split into doublets. For instance for Al K at 1.486keV we get two peaks separated by ~100eV and centered at 1.486keV. Has anybody seen this and is there a cure? ***Alan Nicholls nicholls@uic.edu Thu Oct 13***

Sixteen years out from any contact with Amenex's ETEC Autoprobe, I recall seeing line doubling when the detector was getting overloaded, especially in my hands. The context is that I was trying to write code in TrueBasic to automate the process of setting the wavelength-dispersive spectrometers at the correct angle to detect the X rays coming from specific elements, whose wavelengths are ever so slightly dependent on their chemical surroundings. The software was to scan across the selected wavelength and then select the angle at which the signal was at its maximum. Whenever the detector gain was excessive, the peak would get folded over onto itself, producing the false doublet. Just back off the gain setting. George Langford amenex@amenex.com Thu Oct 13

Are you talking about overflow in the MCA channels? I know we had an issue with that with a Tracor Northern 2000 system from back around 1980. We had a choice of configuration between 24 bits per channel at 1024 channels or 16 bits per channel at 1536 channels. We opted for the second case and could get rollover in extreme cases. However, I think it looked different than what Nicholl's group has run into. Warren Straszheim wesaia@iastate.edu Thu Oct 13

TEM:
mystery light flashes

We are using a JEOL 2100F electron microscope. In the past 2-3 days we can see a sudden "flash" of light on the fluorescent screen along with a faint "click" sound. Rest of the TEM works as normal. This is more prevalent if I meddle too much with brightness knob. Can anyone suggest a reason for this? ***Amit Gupta amit.welcomes.u@gmail.com Tue Oct 4***

I believe you have seen an electric discharge. May be the connection between the screen and the ground is pretty bad. This connection is done through a spring under the screen and the circuit for measuring the current received by the screen. Nicolas Stephant nicolas.stephant@univ-nantes.fr Tue Oct 4

Is there any outlet where I can measure and demonstrate the above—that is, any output like a ground wire where I can safely connect a multimeter and check? If it's Wehnelt contamination, etc., can it be remedied without calling engineers? As there is no maintenance contract, I would like to provide rough estimate to administration before calling JEOL for help. Amit Gupta amit.welcomes.u@gmail.com Tue Oct 4

If you have arcing in the gun, you should get a Geiger counter and check for radiation. If your image shakes, rattles or rolls, then I might go with the gun. I agree, however, with the screen possibility. You may see arcs, but do you see the beam move? Or "flutter" on the screen. Even worse, at high mag, is your focus unstable. If just flashes of light, but not the other phenomena, you might want to vent the camera - viewing area - and check under the screen. Note: Radio Shack has a glass fiber "pen" that is designed to clean contact surfaces. If the spring is attached to ground, you might save your bosses a visit by cleaning the contact area on the bottom of the screen. Note: I have a rule about electron beams. When they misbehave, I always change the filament first. If there is no change, then, depending on age, I might save the filament. Note: Sometime, the gun housing just needs a good cleaning, but with my FEI/Philips Tecnai, there follows a lot of outgassing even after using the sun lamp to start the drying out. Fred Monson fmonson@wcupa.edu Tue Oct 4

Since the 2100F is a FEG system, the gun components differ than those described by some of the responses. I would avoid opening the FEG unless directed by service, or if you decide to replace the FEG tip. Replacement of FEG is not trivial (nor cheap), unlike W or LaB_6, so this is not where I would start... The click you are hearing is worrisome. Is it coming from the gun area (top of microscope)? Or perhaps from the HT tank behind the microscope? Try to determine whether the FEG emission current is fluctuating shortly before or after you hear this click. You may need to set up a camera or screen capture software to monitor the emission current (and vacuum levels!). If the click is coming from the gun area, this is usually indicative of arcing. Bad! Usually caused by lint/dust/particulate contamination in the gun region. This would be unusual for a FEG. Has the FEG been opened in the recent past? If the noise is coming from the HT tank, then you may be experiencing a problem with the tank, which would necessitate a service call. Since this is the 2100 series, it must be relatively young, correct? Probably not an electronics issue, so I'm not sure why changing C2/C3 (brightness) knob would affect operation. As others have indicated, it could be related to charging of the InP screens (don't forget little screen). But click noise means, in my opinion, more sinister problem that you need to find. I hope not! Christopher Winkler microwink@gmail.com Tue Oct 4

Besides the suggestions you have already received, when was the last time the gun and column were baked? The clicking and fluctuating condensers make me wonder if there is a bit of contamination on the condenser aperture. Baking might take care of that. Did this problem start after a particular sample was imaged? One that might cause contamination? Phil Oshel oshel1pe@cmich.edu Wed Oct 5

Thank you everyone for help. The flashes we observed were indeed due to fluorescent screen charging, further action awaits administrative approval! For completion sake here is the summary: Problem: Bright flashes of light were observed with a click sound on fluorescent screen. However, on suggestion from the listserver and further analysis: 1. No sound were heard when screen was "up" 2. No change in intensity seen on CCD camera 3. FE electron gun showed no change in intensity (looking through the window on electron gun) 4. No accelerating voltage fluctuation 5. No vacuum pressure level fluctuation of any kind 6. Flashes were more prominent during alignment (when image intensity will change a lot and screen will be thoroughly radiated due to various intensity wobbling, etc.) 7. Proper corona discharge kind of flash was also seen once or twice (at the edge of screen) 8. Issue disappeared on own for few weeks, now surfaced again Current reasoning: In JEOL JEM screen is held by 3 screws (as told by JEOL engineer) one of which is grounded, looks like ground screw is bit dodgy as the moment. I will let everyone know in case any development occurs. Amit Gupta amit.welcomes.u@gmail.com Mon Oct 24

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