NetNotes

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Selected postings to the Microscopy Listserver from November 1, 2011 to January 1, 2012. 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:
flat embedding molds

Just a quick question: Has anyone used the PTFE flat molds sold by EMS to polymerize LR White (LRW) resin? I would appreciate if you could share your experience on how well the resin consistently polymerizes under these conditions (ideal temperature, time of polymerization, etc.). ***Tami Bogea tbogea@mail.ubc.ca Fri Dec 2***

I sometimes use a similar mold from Pella. I find that getting the Aclar onto the thing without trapping bubbles to be annoying, especially with the shrinkage of LRW during polymerization. Instead, I just overfill the cavities slightly, orient the specimens as desired, and put the filled mold into a plastic dish with sides about 1 cm high (so the bag won't touch the resin) and I put this whole thing into a zip lock bag and fill it with nitrogen (or argon from the sputter coater tank). Just zip the bag mostly closed over the gas tubing, get a stream of gas flowing and let the bag fill, pushing down on the bag a couple of times to purge O_2, then let it fill a bit, pull out the tubing, and press the final cm of zip lock closed. Then load it into the oven at ~58°C for 2–2.5 h. If you put enough resin into the mold that the cavities are joined, they will actually come out as a single strip that is pretty convenient, as getting the blocks out of the mold can be a little tricky because it's not as flexible as the silicone molds. Andy Bowling ajbowling@dow.com Fri Dec 2

In our experience it is better to not fill all the molds with samples. We have to orientate drosophila retina and larvae very strategically. We place our samples into the center 6 wells, then overfill the wells including two extra empty ones on either side of the samples. Press the Aclar film over the samples first then gently lay out the film toward both sides so that the resin pours over into the leftover empty well spaces. The very end well spaces will not polymerize very well, but the ones with the samples will turn out very well, at least for us. We then place the mold into a 60°C oven overnight to two days. So far we have been able to thick section and do fluorescence staining with no problem. Hope this helps. Lita Duraine duraine@bcm.edu Fri Dec 2

We use the PTFE flat mold all the time for LRW resin. We consistently use the protocol suggested in the technical data sheet that accompanies the product. We have always gotten excellent results. Mary Ard maryard@uga.edu Fri Dec 2

I missed the temperature for LRW that was suggested. In that I am doing my first experiment with it in 20 years, I am wondering just what temperature is considered good now. Pat Connelly connellyps@nhlbi.nih.gov Fri Dec 16

LRW has to be polymerized in BEEM capsules or gelatin capsules (air tight) and at 45°C if using for immuno-electron microscopy. It would polymerize even at higher otherwise. Shashi Singh shashis_99@yahoo.com Sat Dec 17

We routinely polymerize LR White at about 60°C under nitrogen gas, so no need to seal the containers. A 2–3 mm deep mold of medium grade resin takes about 90 min to polymerize this way when it's an aluminum mold. When we use any of the plastic moulds it takes about double this time to polymerize, I don't really understand why though could speculate. Resin polymerized at this temperature is fine for immuno work, at least in plant material. You can also polymerize it under UV either sealed or under nitrogen, but our OHS people prefer us not to use UV these days. Rosemary White Sat Dec 17

LR White can also be polymerized for immunoEM down to –20°C if you use the cold accelerator. The manufacturer recommends ~1.5 µL per mL resin, but I've found this leads to inadequate polymerization at –20°C. A more effective concentration is 5–7.5 µL/mL resin. Allow to polymerize overnight at –20°C then put on your bench top and let warm to room temperature. Polymerization of LR White is always improved if the resin is degassed for ~10 min under at least house vacuum before use. If you are cooling the resin for low temperature polymerization, also allow the resin to cool to –20°C before adding the cold accelerator. Richard Fetter fetterr@janelia.hhmi.org Sun Dec 18

Specimen Preparation:
biosafety

We are BL1 level multiple disciplines users facility. Now, there will be a user wanting to do fixed HIV samples for negative staining. Some users feel uncomfortable to hear this. As I know, fixed HIV samples should be OK in our facility. Does anybody have suggestions that we need to follow to handle this case? I have checked our University and NIH website and cannot find useful information. If you have a safety protocol for this situation, could you share it with me? ***Han Chen hchen3@unl.edu Fri Nov 25***

To my knowledge, all studies ever conducted have shown that HIV is inactivated by standard fixation methods. In fact, fixation as low as 0.2% glutaraldehyde is considered to be effective. Paul Hazelton paul_hazelton@umanitoba.ca

I have no experience working with viral samples, but while recently reading some books on EM, I came upon a chapter that treated fixation of virus, and safety. I don't remember exactly what was the conclusion, but I sure remember one thing, that someone managed to rescue viral particles (some, not all of them) from already fixed and embedded sample for standard (I think) EM. So, please check the literature, there should be enough papers on HIV in EM and do contact the authors of the papers. Unfortunately, I can't tell you the book title, because I was checking quite a few (all borrowed) and I don't remember in which one I read it. Josif Mircheski jmircheski@us.es Fri Nov 25

Biosafety issues are not a subject for casual discussion on a listserver; they are to be addressed by the relevant institutional biosafety committee. For Han, I suggest he stop the researcher using the HIV until an approved IBC protocol is in place. This protocol should address how the virus will be handled before it gets to the EM

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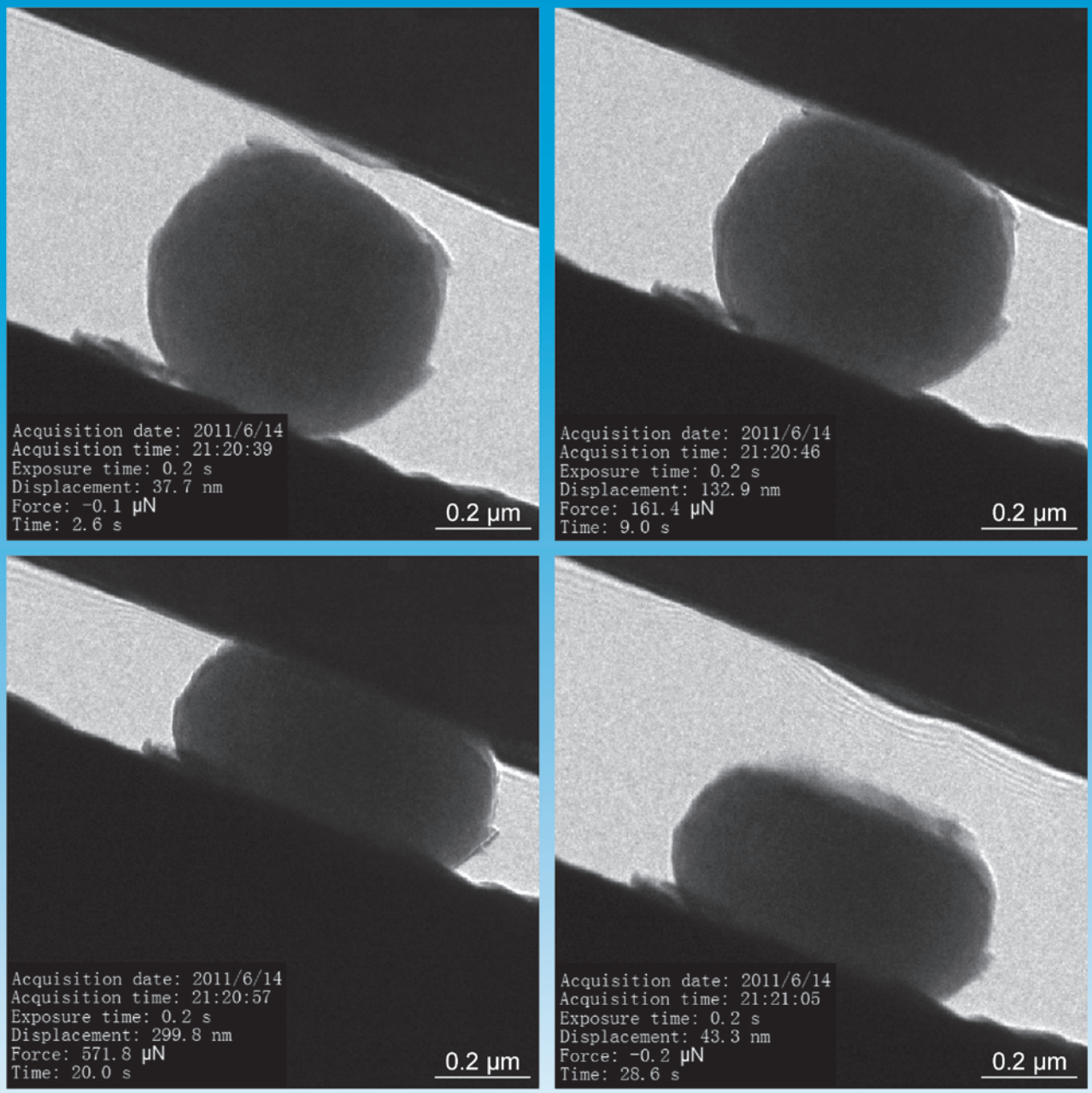

In situ compression of a SiO_2 glass nanoparticle acquired with Gatan ORIUS® 830 CCD camera. A 500 nm amorphous SiO_2 glass particle is plastically deformed using a Hysitron Picoindenter (PI95). The loading rate of 15 nm/s was generated by driving the flat diamond punch seen in the lower left corner of the images. The indentation experiment was coordinated and recorded using Gatan's *In situ* Video Software, a new component of the Gatan Microscopy Suite®. Images courtesy of Prof. Zhiwei Shan, CAMP-Nano, Xi'an Jiaotong University, China.

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lab, how it will be handled in the EM lab and how it will be safely disposed of once it has been imaged. The protocol should address suitable labeling of all materials to be used in the lab, warning signs outside and inside the lab, and relevant personal protective equipment. The biosafety committee is a group of experts who will understand the safety issues of handling biological hazards and will offer the best advice for handling the virus. The other users have every right to be concerned by potential exposure to virus until there is an approved IBC protocol in place. The protocol should also address issues such as how the TEM specimen holder will be sterilized after use so that it can be safely handled by the other users. Again, the safety issue itself may not be important (if the virus is chemically fixed and has been irradiated), but the safety of all users has to be addressed first. An approved IBC protocol should be in place for any lab that is handing viral, bacterial and fungal pathogens: for human cell lines and human bodily fluids. If the lab is routinely handling unfixed human materials it is also advisable for the staff to be vaccinated against the hepatitis b virus. Failure to adhere to approved protocols (or failure to even apply for them) can result in loss of NIH funding and lawsuits. Paul Webster pwebster@hei.org Fri Nov 25

Several have replied with comments about fixation and safety committee rulings. It is my experience that most virus particles are inactivated in seconds upon fixation. Even BSL-3 and BSL-4 particles are inactivated for one hour in fixative (4% paraformaldehyde/2% glutaraldehyde) and then placed in osmium tetroxide vapor to bring them out of containment. My experience is that there is a good deal of information about inactivation of surfaces with various substances, but little to no real information about specific fixatives for EM fixation and analysis. It is possible to do test runs with the fixative on virus particles that were allowed to adhere to coverslips to determine the effectiveness of the fixative on virus inactivation. You could get the PI to do one test with a specific amount of virus solution and treat the coverslip for one hour and then put the washed coverslip into a culture flask to see if there were any remaining viable particles. I have always found that 1 hour in 4% paraformaldehyde/2% glutaraldehyde is a great inactivating solution for pretty much anything. I typically do this at room temperature. If you are still a little hesitant about the time, double it to 2 hours. Robert Pope ropope@gmail.com Sun Nov 27

My message about obtaining IBC approval for handling pathogens was not aimed at being critical of aldehydes as sterilizing agents, but to notify everyone that NIH-approved procedures for obtaining permissions for handling these agents are absolute. Formaldehyde and glutaraldehyde are efficient sterilizing agents that have been used as such for many years. However, there is much more to handling pathogens than making sure the agent has been sterilized. I think that other users will need to know whether the pathogen will be inactivated before it is brought to the EM laboratory, which space will be used, how the grids be disposed of after use, how aerosols will be prevented, and if other users will be notified when someone using the pathogen is working in the shared space? Submitting an IBC protocol should be an easy process but once it is approved it will put the safety protocols in the hands of the PI and give the head of the EM lab a level of control that will be needed if the people handling the pathogen are not as careful as they should be. In the case of HIV virus, the risk of cross-infection is very low even if the particles are not inactivated with aldehyde or bleach, but the risk is still there. Check with your IBC before doing anything. Paul Webster pwebster@hei.org Sun Nov 27

I must agree with Paul on this matter. HIV poses real safety issues and working without a precise protocol and strict rules is irresponsible. If someone fixes the virus with aldehydes this should be done in a fume hood with strict safety procedures. Is the fume hood BSL-2? What kind of filter does the hood have? Who has access to the hood? Even after fixation you should not treat it as a BSL-1 material. Let me remind you that one of the principles of BSL-2 is containment, meaning only authorized personal has access to the material and everything is precisely labeled. As Paul said, everybody has the right to work in a secure environment. You must assure that untrained personal never come in contact with this material. Never meaning not even once, by mistake. Stephane Nizets nizets2@yahoo.com Mon Nov 28

We did our HIV research in a P3 level laboratory and fixed all HIV-infected cells with 3% glutaraldehyde in cacodylate buffer, post fixed in 1% OsO_4. And we used 9% bleach to clean up. Barbara L. Plowman bplowman@pacific.edu Tue Nov 29

Immunocytochemistry:
nanogold

A colleague of mine is trying to use immuno EM with nanogold particles, but at the magnification that she needs, the 5 (or 10 nm) gold particles cannot be seen clearly. Could someone suggest a protocol (homemade better) for gold particles enhancement? (A protocol that uses silver, nickel, or anything else.) There are some kits already available, but she would prefer using her own reagents. ***Josif Mircheski jmircheski@us.es Tue Nov 15***

Though commercially available enhancers (e.g., from AURION) work very well, the best (most sensitive) enhancement still can be achieved (at least in my hands) by the good, old homemade Danscher silver-intensification (Danscher G, Nörgaard JO 1983 Light microscopic visualization of colloidal gold on resin-embedded tissue. *J Histochem Cytochem* 31: 1394–8). In my hands, 0.8–1.0 nm Au is enhanced in a 2-step intensification (6 minutes and 8 minutes); Quantum Dots (4–5 minutes and 5–7 minutes); all at 37°C. For larger particles 7 plus 11 minutes could be applicable, but unspecific background will also rise. Peter Heimann peter.heimann@uni-bielefeld.de Tue Nov 15

I generally prefer do-it-yourself in regards to things like this but would go with the kit approach in this situation. I am a big fan of Nanoprobe's gold (not silver) enhancement kit. It is much easier and more reproducible than the silver enhancement kits or "homebrew" approaches I used in the dark ages. Tom Phillips phillipst@missouri.edu Tue Nov 15

Image Processing:
3D reconstruction of fluorescent images

We would like to take serial sections (20 microns thick) of rat spinal cord (up to 1 cm long), label them with a bright and specific fluorescent label, acquire images (we can use either a widefield or a confocal microscope) and then reconstruct the data in to one, 3D structure. We have the sectioning, staining and acquisition parameters/options under control, but do not have any software capable of reconstructing the resultant images. Does anyone have any suggestions as to what software to use and where to get it? ***Emma King emma.king@nottingham.ac.uk***

I have no experience of doing something like this with so many sections (5,000) in fluorescence. The problems are likely to be the same as those faced by people using EM to reconstruct large (relatively speaking) volumes in 3D—namely alignment of the images and distortion caused during sectioning and mounting. I don't know

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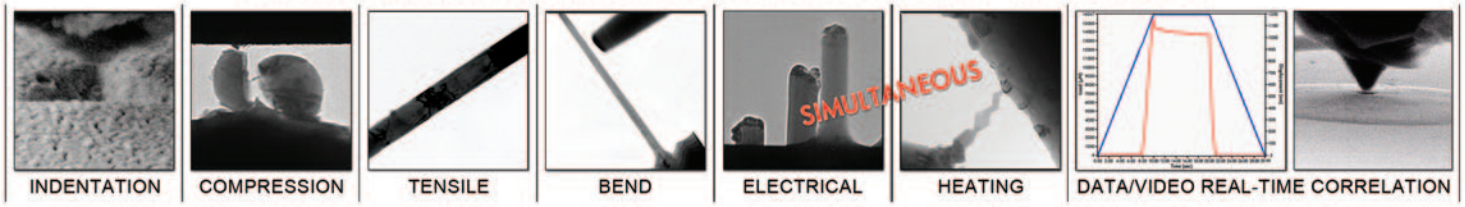

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what sort of resolution you need, nor how to solve how the labeling would be done, but would some form of imaging directly from the tissue block before sections are removed (or milled) be possible? The equivalent is done with block-face scanning EM to solve the same problems. Something along the lines of: http://onlinelibrary.wiley.com/doi/10.1002/jemt.20491/pdf. Or you could find a way of capturing and mounting the sections all at the same angle without introducing much distortion. Or you can use software to overcome these. http://onlinelibrary.wiley.com/doi/10.1002/jemt.20829/pdf. Although the following page is a bit old, it may have some useful links: http://www.wadsworth.org/spider_doc/sterecon/ssrecn.html. Ben Micklem ben.micklem@pharm.ox.ac.uk Mon Nov 21

This sounds like a great project! First I strongly recommend that you use a confocal with settings for a small optical section in order to give you enough data for reasonable *Z*-resolution in your final reconstruction. I typically used 0.5 µm steps with a 63× and should have used 0.2 or 0.3 µm steps. The software I have used most is Imaris from Bitplane. It is robust with useful features for neuro studies and they have good support. The cost is high with all similar software. If you try to use open source software you will spend many hours and days getting it all working for your application. Larry Ackerman larry.ackerman@ucsf.edu Mon Nov 21

We use Perkin Elmer's Volocity 3D visualization software for this, but it is expensive. Others may use Fuji or ImageJ 3D plug-ins, which are free to use, and the confocal list-server replies have mentioned a few of those options. Freebie Fuji's not really any different to Freebie ImageJ for 3D work as far as I can see, and as you'd probably use a 3D ImageJ plug-in like Deconvolution Labs so you may as well stick to ImageJ if you find a suitable ImageJ 3D plug-in. Fuji's integral strength seems to be more stitching together 2D images from motorized XY stage raster scans to recreate whole tissue sections [which Photoshop CS4/5's Photomerge can also do well provided there's overlap]. ImageJ plug-in Deconvolution Labs is quite impressive for 3D rendering, although you might have to follow on-line help to avoid the persistent memory crashes. Its PSF calculations seems similar to Volocity's in that you don't have to input much to get going. The only thing I'm sure about is adding together images to get the *Z*-sequence—if there's no ImageJ freebie for this I suppose any basic video editing package should be able do this, such as PC Pro's A listed Sony Vegas Movie Studio HD Platinum Suite 11. It shouldn't be a problem though other than you need to input your *Z* distance between sections/optical slices. Recreating 3D *Z* stacks is very memory intensive and both freeware ImageJ and expensive Volocity can struggle with frequent crashes due to system memory problems. With Volocity that's hopefully overcome with Windows 7 64-bit, loads of system RAM memory [8Gb] and a fast 3D gaming card, as the codes optimized for that—plus Volocity's strength has always been Improvision's/Perkin Elmer's help desk, where the answer to your problem is generally minutes away [hence the reason I suppose why Volocity costs serious money]. Although Volocity is expensive it's possible someone has a license for the program within the university, so it would be worth checking that out [Perkin Elmer support who produce Volocity can advise]. If you find a Volocity workstation, contact Perkin Elmer's Volocity support regarding your spinal cords as they will be able offer advice on this. I've actually not tried adding together single *Z* slices or multiple *Z* stacks from multiple sections using Volocity, as here we always create *Z*-stacks from one specimen and that's done automatically by our acquisition software with a *Z* motorized focus and saved as a single Volocity compatible file [it can read most files a microscope PC is likely to create]. Plus our Volocity version is out of date and the latest may do more [we hope to upgrade to v6.0 this year]. It should be pretty easy to add folders of images or image sequences to the library though [it's all drag and drop]. I only mention Volocity as we have the software, there's also suitable 3D plug-ins for other commercial packages like MetaMorph, Image Pro Plus, Imaris and Huygens Software (SVI) to name a few, so check if nearby colleagues in your university have these as well. Even if you end up using ImageJ 3D plug-ins, it's useful to see how the result compares with commercial 3D visualization and restoration software. X-from our point of view the only thing Volocity lacks is a neurite outgrowth tree-measuring app, although our MetaMorph Offline has a Neurite Outgrowth module and there are similar versions available as plug-ins for ImageJ [the action of which we can usefully verify with our MetaMorph license]. Bitplane's Imaris is also very strong in the 3D neurite measurement area with their Filament Tracer software, and that seemed very impressive when demonstrated to us recently. We also have NISElements and its EDF function [extended depth of focus]. The EDF app also takes a wide-field [not confocal] Z stack through a tree like structure [neuron-type structures in brains or blood vessels in our case]. This time though it doesn't create a 3D image but analyses each image and selects only the in-focus parts. It then adds all the focused bits from every image and fits them into a single 2D image—squashing the 3D tree into a single focused 2D photo of the tree as it were. So you want your 3D tree structure lying across the field of view for this to work well. If you are lucky enough to have a Volocity license you probably won't be so interested in ImageJ's versions as Volocity can do most that you would require, such as deconvolution (Restoration), Quantitation (tracking objects and volume/distance measurements), and Visualization (4D videos). So others on the Confocal/Microscopy list-servers who use ImageJ for this should be able to advise on any 3D/4D plug-ins like Image5D (that looks like it can volume quantitation), FluoRender or Hypervolume—plus there's plenty of help for ImageJ online. We use ImageJ here as well as MetaMorph v7.7 here, but largely for 2D image analysis as there's conveniently no license restrictions for ImageJ. Keith J. Morris kjmorris@well.ox.ac.uk Tue Nov 22

EM:
refilling vacuum pump oil

We have a JEOL 100S electron microscope and now the level of the rotary pump (RP) oil is low. Does anybody know whether it is necessary that the microscope be turned off to refill? ***Armando Obregón Herrera yodapesister@gmail.com Thu Nov 3***

We top off our RP oil pumps while they are running. This should not be a problem. The filler plug is at the top of the unit and you can just carefully pour oil in. However—when was the last time you changed the oil? We replace our RP oil yearly. Shut down TEM and replace oil, you may want to replace the belt at that time, then power all backup. Roseann Csencsits rcsencsits@lbl.gov Thu Nov 3

EM:
unexplained vacuum problem

We just experienced a rather unusual issue for the second time and now we'd like to investigate it further. We have a Cameca SX100 using a LaB_6 source and equipped with a Gatan MonoCL3 cathodoluminescence imaging system. During CL acquisition (during the last two attempts), the gun vacuum inexplicably increases (ion pump shuts off, no vacuum reading in gun), shutting off the beam and forcing us to open the gun valve to position 3 so that the gun system can get back down to the 10^{-6} Pa range. During this time, the vacuum in the chamber does not seem to change and remains at the 10^{-4} Pa level— so we don't think that it is a problem with the CL mirror arm. It seems to

occur when the Digiscan system takes over control of the probe. Does anyone have a similar experience or thoughts on why this would occur? ***Dan Ruscitto ruscid2@rpi.edu Wed Nov 23***

Any chance that the ion pump might be at the end of its lifetime? I had this problem at my LaB_6-SEM just some weeks ago. The pump had not been able to getter all the gas from the hot cathode environment after approximately 30 minutes of work and that accelerated like an avalanche, the pump finally becoming very hot. Stefan Diller stefan.diller@t-online.de Wed Nov 23

TEM:

Gatan tilt holder

We use a Gatan double tilt holder. There have been repeatedly a couple of accidents over the years where a user managed to bend the front tip at where the specimen cradle is. The front tip part appears to be aluminum and the sample cradle connects to the holder via two tiny brass pins. Allowing the space for cradle and notch clearance for x-ray escape, the main holder part is apparently very weak. The accident sees the rod tip being bended exactly at the weak points where the two pins connect the cradle and serve as pivot points. Unfortunately, none of my users owned up to it. We struggle to figure out the causes of the accidents, so we do not know what to improve to avoid it. We certainly do not want to repair repeatedly for the same problem. We would appreciate any ideas or advice on how this could happen, if we can repair this by ourselves, and how to prevent it in the future. ***Z. Zhou z.zhou@lboro.ac.uk Fri Nov 11***

Being a user facility with more than 50 TEM users, I can sympathize with the problem of broken double-tilt holders. We have had this occur several times in the past. One solution has been to fabricate a clamping mechanism that holds the 2-t holder securely on the bench-top stand during the loading of sample. It is most important to prevent any rocking/rotating of the holder during loading as this produces stresses on the pivot pins, causing breakage. The second important solution is to provide good support under the sample cup during sample loading. Gatan and newer FEI stands typically have the holder tip extending out into free space. We reverted to using the older FEI/Philips stands that provide support for the holder tip. Since introducing these changes, we have had no breakage during the loading step. I can offer no help for breakage due to heavy-handed or slippery-fingered users, other than suggesting my method of applying the "user alignment tool"—a large wooden mallet. Roger A. Ristau raristau@ims.uconn.edu Fri Nov 11

Sigh. The anonymous cack-handed klutz strikes again. Someone recently mounted something large on our VP-SEM stage and rammed it up into the BSE detector, producing a hairline crack across 2 quadrants. It took us a while to figure out what was wrong; ramming something into the detector was outside our thought space. And of course, no one owned up, though we are pretty sure when it happened, that is, who the culprit was, because we could see when image quality got really bad. It is hard to believe that a couple of users accepted this poor image quality without comment. One way to try to track this down is to compel all users to comment on the status of the instrument when they start their session. This is quite good for figuring out who gets oil all over objectives, for example. For serious damage, you end up paying for it no matter what, and we have compelled all of our SEM users to be retrained, and set the stage stop way down. . . . You could insist that all of your double tilt holder users be retrained, and have a policy that they report on its status at the beginning of each session and see if that helps. Even if you don't track down the culprit(s), you might reduce the number of incidents. Rosemary White rosemary.white@csiro.au Sat Nov 12

We experienced exactly the same thing. The only way of preventing it is to have a competent user load the samples and insert the holder. Fixing it yourself is very difficult; the tip typically needs replacing when it is bent like this. I am wondering if it is not possible to fabricate the tip from titanium to increase its durability. I suspect that that would increase the replacement cost considerably. John Mansfield jfmjfm@engin.umich.edu Sat Nov 12

Gatan does offer a titanium tip replacement for their double tilt holders. I had to have this done to repair one of our specimen rods that had achieved a rather impressive 25-degree bend at the pivot points. The main contributing factors were using a specimen rod cradle that did not clamp the rod and excessive muscle reflex when the rod started to tilt. I now only use the Gatan clamping stand when changing specimens in their specimen rod. When I had the repair done about 10 years ago, the cost was ~$3500, which was about $1000 more than the standard tip. Philip Flaitz flaitz@us.ibm.com Mon Nov 14

Regarding "how to prevent this in the future", another option that I've seen work is to require all users of your double-tilt holder to "re-qualify" on its use. Basically that would mean the next time they sign up for instrument time and plan on using the holder, they need to be observed by you. While some users will be on their best behavior and mask holder-breaking practices, many times this exercise serves to demonstrate what users are doing right, and also what they're doing wrong, and can sometimes expose the practices which cause the breakage to begin with. This is not the same as retraining everyone, since retraining is interactive and can mask the problems, which simple observation/"re-qualification" has the opportunity to expose more issues. John Papalia jpapalia@papalia.net Mon Nov 14

I have the same DT Gatan Stage since 1990. We have had repairs to it many times over the years. From your description and my experience with this your email suggests that the user pulled the holder or bent it sideways from the goniometer before it cleared the insertion hole. I tell the students to withdraw the stage totally away from the microscope goniometer stage before moving away from the microscope. Fred Pearson eoptics@mcmaster.ca Tue Nov 15

EDX:

spectrum of silicone rubber

I am having trouble with interpreting an EDX spectrum of a silicone rubber sample. The elements detected are carbon (8,295 counts), oxygen (13,483 counts), and silicon (90,549 counts). So, as you can see, I have a really big silicon peak with oxygen being a moderate sized peak and carbon small. The problem I am having is that I am getting a small (910 counts) peak at 2.235 keV that doesn't really line up with any specific x-rays on any of the charts and does not appear to be an escape or sum peak for any of the elements present. The closest identification would be either thallium or mercury, but it is not likely they would be present in the rubber. I do get a good-sized silicon sum peak at 3.465 keV (1136 counts). The question to the list is whether or not this peak is an artifact or perhaps a silicon x-ray peak that is not listed on the charts. I am operating IXRF EDS 2008 computer software. ***Don Kierstead donk@ardl.com Wed Dec 7***

The peak you see is very close to O+Si sum peak, and I strongly believe it is the O+Si peak. You can try to decrease intensity of excitation beam until Si sum peak disappears, then O+Si peak should disappear also. Vladimir M. Dusevich dusevichv@umkc.edu Wed Dec 7

This is pile-up: Si+O. Frank Eggert eggert@mikroanalytik.de Thu Dec 8

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