NETNOTES

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Selected postings from the MSA Microscopy Listserver (listserver@msa.microscopy.com) from 12/10/04 to 2/10/05 Postings may have been edited to conserve space or for clarity.

LM – TIRF

What is the single advantage provided by TIRF microscopy that allows one to view single molecules? <springpaard@yahoo.com> 11 Dec 2004

Total internal reflectance microscopy (TIRF) provides resolution on the order of tens of nanometers in the axial dimension. This is because of a phenomenon that occurs when the rays of excitation illumination are glanced off a reflective interface (an interface between differing refractive indices) at an angle greater than the second refractive index will accommodate (in other words, a much higher numerical aperture than is possible to attain in the second refractive index). When light is reflected off an interface in this manner, a very small portion of the media past the second refractive interface is illuminated by evanescent waves from the illumination source. The intensity of evanescent waves decays very rapidly so illumination is only intense enough to excite fluorochromes to a depth of a few tens of nanometers. This provides a means of determining that bright spots are localized to very near the interface with a resolution that surpasses that of conventional diffraction limited illumination schemes. An example of this would be viewing the distribution of labeled proteins in the cell membrane of cultured cells adhering to a coverslip. To answer your question more directly, the big advantage of TIRF is very high depth resolution at the coverslip/sample media interface. That is the single big advantage. This means a very small sample volume can be monitored and investigators can monitor molecules moving into and out of this small volume. From these observations, calculations that provide insight into molecular concentrations, whether or not the movement of molecules appears to be random, correlation of spatial relationships between (and colocalization among) different types of molecules and other parameters can be conducted. Karl Garsha <garsha@itg.uiuc.edu> 13 Dec 2004

LM – GFP and Autofluorescence

I have a user who is trying to look at GFP expression in plant leaves. We are having a hard time with the fluorescence from chlorophyll masking everything else. Is there some way to quench the chlorophyll, without killing the GFP? Shea Miller <millers@agr.gc.ca> 09 Feb 2005

Try keeping the excitation power as low as possible. When we image GFP in leaves, the chlorophyll starts getting in the way when it is emitting enough light that this emission spills into the GFP channel. R. Howard Berg <rhberg@danforthcenter.org> 09 Feb 2005

On a confocal, you can collect chlorophyll autofluorescence above about 650 nm and if you collect GFP in a narrow band in the green, you should be able to detect the "pure" green from GFP separately from any chlorophyll autofluorescence. You could also subtract the red from green, or use whatever dye separation package is available on the microscope. While chlorophyll is not supposed to auto fluoresce in the green, we routinely see some fluorescence there. If your chloroplasts are "suffering" you'll get more auto fluorescence in the green and yellow. Treating the tissue with DCMU should reduce chlorophyll fluorescence; it's a herbicide that attacks one of the quinones in the photosynthesis electron transport pathway. Not sure if it has other effects in the cell. In contrast, if I remember correctly, PCMBS will enhance chlorophyll fluorescence. If you have narrow-band emission filters on a regular fluorescence microscope, you could collect chlorophyll autofluorescence using a Texas Red filter, and GFP with a narrow-band green emission filter, and overlay them. If GFP is there, you should see it. Rosemary White <Rosemary.White@csiro.au> 10 Feb 2005

SEM - Nematodes

I received some nematodes for SEM. They are preserved in 96% ethanol. I have no experience with this sort of organisms. Can anyone please tell me how to proceed? Renaat Dasseville <renaat.dasseville@ugent.be> 15 Dec 2004

If you go to our website at www.wormatlas.org you will see a listing for "Anatomical Methods" for the study of the nematode. In this listing, there is a detailed protocol for doing SEM on the nematode, or even on dissected organs from the nematode. Other portions of our website will help to inform you about the various tissues that you might encounter, but you may especially want to read the "Cuticle" chapter in the Handbook. David Hall <hall@aecom.yu.edu> 15 Dec 2004

SEM – Microemulsions

I need your help in imaging microemulsions of oil-in-water/water-in-oil with oil drops of 10 to 50 nm size. Do you have any experience in SEM/ESEM/CryoSEM imaging of such objects? Inna Popov <innap@savion.huji.ac.il> 02 Jan 2005

If you have access to an SEM with a liquid nitrogen-cooled cryostage and a way of plunge freezing samples and transferring them frozen into the specimen chamber, this would be a good way to do it. I have imaged foamy liquids with great success like this, and the process was very simple. Briefly, the liquid to be imaged was shaken violently to form the bubbles, and then a drop of the foam was put on the specimen holder and plunged into liquid nitrogen. With my cryopreparation unit, I took the sample out of the LN_2 bath under vacuum and transferred it to the fracture and coating stage of the unit. Using a built-in pick, I fractured off a piece of the frozen foam, and then coated the sample with gold before transferring it under vacuum to the SEM stage. Micrographs were taken with no problems. This was actually one of the easiest cryo specimens I ever worked with, and I expect yours would work much the same way. My only concern might be the size of the droplets you want to image, since cryoSEM, in my experience, doesn't have the resolution of "normal" SEM work. Randy Tindall <tindallr@missouri.edu> 03 Jan 2005

SEM - Wobbler Adjustment

I am trying to understand the principle of the focus wobbler adjustment for an SEM. Can anyone please help me? I found an earlier thread in the MSA achieve but couldn't find the article by Le Poole and other related articles.. I understand that the focus wobbler in an SEM is meant for aperture alignment. How can I tell which alignment (the "x" or the "y") to adjust? T. T. Tan <tttan@simtech.a-star.edu.sg> 29 Dec 2004

If (1) the final aperture is misaligned, or if (2) the beam is astigmatic, it becomes evident as you focus above and below exact focus. This is what the "wobbler" is supposed to help you with; that is, to continuously focus above/below, while you make the proper adjustments. Some SEM operators, maybe most, have learned not to use it. Depending on the sample, or the degree of the problem, it may be easiest to simply make the adjustments until the best focus is achieved. While the wobbler is enabled, if the image shifts during focus (left-right, up-down, diagonally; twisting is normal), then the problem is the final aperture. Beginning with either aperture adjustment (x,y), I usually adjust the wrong way first to make the problem worse, and then I adjust through and past the problem, to make it oppositely worse, and then I come back and back again until I minimize the problem. Do the same with the other aperture adjuster. If the image does not move, but out-of-focus has directionality (as you describe), the problem is astigmatism. The adjustment of the "stigmator" is a bit more difficult to describe. Additionally, there are 2 different types of stigmators used by different manufacturers. One type uses "x-y" adjusters and you would approach correcting the problem similar to above. However, if the stigmator is the "rotation-amplitude" type, I find the best approach is to make the problem worse with 'amplitude' and then minimize the problem with 'rotation". Re-iteration may be required, and keep in mind that your problem may be a combination of (1) and (2). Michael Shaffer <michael@shaffer.net> 29 Dec 2004

It's true that correcting astigmatism is difficult to describe without actually having someone observe it. The approach I take, with pretty decent success, is as follows: Find an area with lots of non-directional detail (i.e., straight edges are bad while "sandy" looking areas are good). Throw the image out of focus until a "smearing" effect is noticed, then go through the focus point and observe that the "smearing" is now shifted 90 degrees from its first orientation. This is definitely astigmatism when this is observed. Using the focus knob, adjust the image until you are in between the "smearing" parts of the focus range. In other words, the image should now be free of directional distortion, but will probably be somewhat out of focus and if you turn the focus knob significantly in either direction, the smearing will return. Now use one of the stigmator controls, either x or y, and turn slowly, using it as a "fine focus control". The image should appear to get blurrier or sharper; make it as sharp as you can. Then do the same with the other stigmator control. Repeat the process a couple times, then go back to the focus knob and adjust it back and forth through the focus point. If astigmatism has been corrected, the image will get blurry on both sides of focus, but should not show any directional "smearing". "Smearing" is the effect you get by putting cooking oil on your hand and wiping it in one direction on your kitchen window. The view of your yard is now blurred and distorted in the direction of your application of the oil. Make note to clean the window before your significant other sees it; explaining this as an education tool is non-effective. Randy Tindall <TindallR@missouri.edu> 29 Dec 2004

SEM - Lubricants

Does anyone out there have experience in SEM on lubricants or grease? I need to do SEM on nanoparticles in lubricates as well as various states of lubricates growing old. Stefan Diller diller@stefan-diller.com 10 Jan 2005

I think you may need to do frozen imaging or cryo-SEM. It might work doing a freeze-fracture-etch and then image with a high resolution coating. Gordon Vrololjak <gvrdolja@nature.berkeley.edu> 10 Jan 2005

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I have experience looking at greases and lubricants in the SEM. One such request had nanoparticles in the grease. Unfortunately, my results aren't promising. I don't really see how a lubricant can be imaged without using an environmental chamber SEM. I had limited success when the lubricant thinned out enough to where I can begin to see the nanoparticles. It also helped that the particles were tungsten-based for backscatter image contrast. Resolution was poor. EDS analysis was somewhat doable. Can you perhaps separate or concentrate the particles by using solvent and centrifuge techniques before imaging? This may help. Stu Smalinskas <smalinskas@yahoo.com> 10 Jan 2005

I do have a cheap and cheerful way to overcome your problem. One feature however may make my explanation null and void! You need a microscope that has a manifold directly pumped by a diffusion or turbo pump, not a manifold that bleeds the specimen vacuum to the gun. You also need a backscattered electron detector. In this procedure, we are taking advantage of a poor vacuum bleeding away surface charge and reducing media evaporation from the specimen. If you have a manifold system and a backscattered detector, the following works very well for up to 20 minute working periods. Take a rubber bung/stopper that will fit into the pumping line at the rear of the specimen chamber, freeze it in liquid nitrogen and drill a 0.5 mm hole in the bung/stopper. Prepare your specimen and place it in to the microscope at the same time fitting the bung/stopper in to the pumping line. Pump down and switch off the SE detector bringing the BSE detector into play. Use the BSE detector to observe the specimen in the "poor vacuum" environment that you have created. I have used this technique many times; the only drawback is that the many manufacturers took us away from a decent vacuum system when they decided to pump the column through the specimen chamber, rather than pumping the microscope through a manifold. So users of older microscopes have the cheapest possible "VP System", but those caught in the middle era in SEM development miss out on this one I am afraid! Steve Chapman <protrain@emcourses.com>

STEM - Beam Induced Contamination

I am a new TEM/STEM user. My machine is operating at 200kV. I am interested in looking at metal nanoparticles, diameter <20 nm. I have deposited such particles (Au) upon carbon support films. I am having many problems with beam induced contamination of the carbon support, making imaging of the nanoparticles impossible with STEM. I am investigating ways of cleaning the supports before depositing nanoparticles. I wonder if such contamination problems are common with STEM and would be interested in possible solutions to the problem. I am currently investigating cleaning with organic solvents and heating or irradiation with UV. Neil Young <neil@young8696.freeserve.co.uk> 21 Dec 2004

There are those much better qualified to answer this than me but I'll give an answer as a 'neighbor' with some experience of it. Yes, contamination is a problem with STEM. It is with all small probe modes, especially as you use higher and higher

beam currents. You will see contamination in a FEG STEM when your specimen looks OK in every other machine. First you need to identify the source of the contamination. It is, as you say, most likely to be from your specimen. Do other users have contamination free STEM sessions with the same holder? If so, then you can rule out the column or holder cleanliness, if not then maybe there is a problem there. If you 'flood' the specimen with electrons, does it reduce the contamination rate for a while? If so, then the major contamination source is your specimen, if not, the contamination is coming from the vacuum. To flood the specimen, use a large C aperture and spot size and keep the beam on an area larger than a grid square (~200 μm dia) for 15 minutes. Check the contamination rate in the center of the area before and after flooding. If it is the C filmed grids giving rise to the contamination, we have found that annealing them in a clean vacuum is a very efficient way of cleaning them, about 200°C for 10-15 minutes is usually enough. The Cu tends to get annealed if the temperature is too high; not a real problem, it just makes handling them more difficult. Plasma cleaning can also be used, if you have access to a plasma cleaner, but it will also dissolve your film. Ensure your method of depositing gold is clean or else you've wasted your time cleaning the C film. If it is a diffusion pumped vacuum, then it should be trapped to prevent backstreaming of oil. If it is a chemical or solvent, then it needs to be clean and pure, fresh from the container into cleaned glass and not from a plastic wash bottle. Ensure your method of storing specimens is clean. Avoid contact with plastic containers - gelatine capsules should be thrown away and never used. Keep specimens under clean vacuum or in a dry container. If no one has clean specimens and flooding does not improve the contamination rate, it is probably your specimen holder (clean it properly) or column (bake it out) causing the problem. If the contamination rate from your sample is small, you may be able to use the flooding method to prevent contamination build up for long enough to run your experiment. Ron Doole <ron.doole@materials.oxford.ac.uk> 21 Dec 2004

I'll echo Ron Doole's comments. You can also access the information on Plasma Cleaning at this WWW Site. http://www.amc.anl.gov/Docs/ANL/TechTrans/PlasmaCleaning.html There are some articles and a book chapter I wrote at that site. A number of vendors market plasma cleaners. Nestor J. Zaluzec <zaluzec@aaem.amc.anl.gov> 21 Dec 2004

I would like to add my two cents into this discussion regarding carbon contamination. This is in regard to observations that I have had using carbon films, FEG TEMs, and plasma cleaners. I have anecdotal information on carbon films and plasma cleaning of coated samples that I would like to share. General Claim: The type of carbon that you have is going to play a role in "contamination" effects and the effectiveness in carbon removal with plasma cleaners. When I was working at Wright Patterson Air Force Base, we were looking at pulsed laser deposited (PLD) diamond-like carbon (DLC) films. First, it is well-known in the literature that the way the DLC films are prepared and their inherent chemistry plays a major role in their tribological properties as well as their reaction to environmental factors. The films that we grew by PLD showed a layered pattern of alternating regions of amorphous DLC that varied in the ratio of sp3 to sp2 bonds, i.e. the darker bands were more diamond-like and the lighter bands were more graphite-like. When a focused FEG probe was used to acquire EELS spectra, the dark bands would show all sp3 bonding initially, but immediately start showing or growing the sp2 structure during beam exposure within seconds. The light bands would do it also, but the rate of build-up was comparatively slower (never measured - only observed). Since the films were grown on silicon substrates, I prepared these samples by the small angle cleavage technique. This gave great, very thin carbon films, and SACT is very good for minimizing, if not eliminating, contamination problems. If the beam was not converged on the PLD-DLC coating, but was used in the normal imaging mode, no contamination build-up was seen, regardless of how much time the sample was under the beam. If the converged beam was moved to the substrate, the beam could be in position for up to 5 minutes with no noticeable contamination. I concluded that the beam was converting the carbon type when converged on the coating and that it was not contamination. When I first came to PPG, we had a JEOL 1200EX that operated at 120 kV. At this voltage, the glass is softened because of the energy that is deposited in the glass. When the samples were taken to a 200 or 300 kV machine (outside lab with FEG or LaB6), we did not see the softening effect. To help the problem when imaging at 120 kV, I found that a very light evaporated carbon layer helped dissipate the heat as well as eliminate charging effects. The evaporator was a diffusion pumped system and pretty dirty. I worried about contamination problems in the FEG machines if I used the samples coated with this carbon in these better FEG machines. To help minimize charging in samples taken to other sites with FEG, I developed a way of sputter coating carbon films destined for use in the good machines with my ion mill. Well the situation arose where I didn't have one of the "clean" carbon sputtered samples and only had the 120 kV carbon evaporated films. I thought that I could plasma clean the samples for a very short time and partially remove the carbon and have a light, but sufficiently conducting film for the FEG use. What I found was that the evaporated carbon resisted removal by plasma cleaning. Playing around a bit, I found that the sputter coated carbon films could be removed by plasma cleaning. There are other behaviors of carbon films with plasma cleaners that were written about in several publications in which I was a co-author. Those publications are available at the South Bay Technology web site for downloading as was mentioned by another posting. I think that an image of the SACT prepared PLD-DLC film on silicon with the EELS spectra is also available from their site. My point in submitting these observations is that the behavior of the carbon films both under the beam and with plasma cleaning will differ depending on the state of the carbon that is determined by the deposition parameters.

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Scott D. Walck <walck@ppg.com> 22 Dec 2004

I would like to make the following comments: a) the efficacy of the cleaning depends on the power being used for the cleaning, with 100 watts being much more effective than 10 watts. The higher power units would also be much more aggressive in terms of etching away a carbon filmed grid whereas 10 watts, so far as I can determine, would not etch away a carbon filmed grid. Units described as plasma cleaners generally operate at 10 watts or less, plasma etchers at 100 watts or more. One can clean with an etcher in some circumstances, with oxygen, where there are no carbon inclusions or other carbonaceous domains in the sample. One would not want to clean with Ar at 100 watts since those conditions would argon-etch the rod and holder and maybe the sample itself. b) One could use a SiO2 filmed grid in many of the instances where one would be using a carbon filmed grid. One of the main reasons why one would prefer to use a SiO2 filmed grid is that the grid can be "cleaned" with pure oxygen without any fear of the substrate being etched away. The SiO2 filmed grids will generally withstand a more aggressive cleaning at higher power levels. Exposure to the higher power level generally results in a longer contamination-free observation time. c) We have never seen instances where some carbonaceous contamination formed in a TEM on a TEM foiled sample can be removed this way and other contamination could not (because it was a DLC). Charles A. Garber <cgarber@2spi.com> 25 Dec 2004

We have tried SiO2 films in our lab for FIB liftout for just the reason that Chuck mentioned, i.e., that we should be able to then plasma clean without losing the sample. We have tried both bulk and lacy SiO2 films. What we found was that when the liftout membrane is sufficiently non-conducting, the membranes charged-up and flew off the grid. Very distressing! We tried to cure this by using lacy carbon grids and Cr coating them, but the stress of sputtered Cr broke the membrane. Carbon coating is not an option since plasma cleaning will remove the carbon. We have stayed with Formvar membranes and adjusted our plasma cleaning to allow us to minimize contamination without destroying the Formvar support film. This was originally driven by the need to plasma clean polymer based low-k dielectrics for chip fabrication; as a side benefit, we found that we preserved the Formvar support film. Using a reduced O2 content in Ar, and reduced power settings, we can do multiple iterations of plasma cleaning. Typically, we find that 20 seconds is sufficient to allow EELS analysis without causing measurable C contamination. I have been able to do three such cleanings on a typical grid without losing the specimen. Philip Flaitz <flaitz@us.ibm.com> 30 Dec 2004

TEM – Nanoparticle diffraction

I am a beginner in using TEM. I am currently investigating nanoparticles of 20 nm and below. I have difficulty in getting their diffraction pattern in TEM. Tay Yee Yan <one_twinklestar@yahoo.com.sg> 18 Dec 2004

One problem you might have is that such small particles

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make up a very small fraction of the area seen in even the smallest selected area aperture you have in your scope. Unless the particles constitute a reasonable fraction of the scattering, the pattern will be lost in the power spectrum of the substrate in the selected area. My advice is to use the thinnest possible support film, make that film from low-Z material (carbon is probably best), increase the concentration of nanoparticles in your prep, and put a very small aperture (5 or 10 μm) in the SA aperture holder and use it to restrict the area. You will probably see only diffraction rings, since the hoped-for many particles in the selected area will not, in general, be oriented in the same direction. Bill Tivol <tivol@caltech.edu> 20 Dec 2004

Are you certain that your particles are crystalline? Properly recording and interpreting the results aside, E.D. is simple enough that anyone with an instruction book or a colleague nearby can work the controls of a TEM to get some sort of a diffraction pattern on the screen. If you illuminated some of your particles with an electron beam in a TEM set in diffraction mode, and all you see is a single bright spot centered on a featureless, diffuse background, it could be that your particles are amorphous (or nearly so). Ron Anderson <microscopytoday@tampabay.rr.com> 20 Dec 2004

This is not exactly a beginner's problem and is, in fact, quite hard. If you want powder diffraction patterns, then put a large SA aperture in and hit Diffraction, easy enough. If you want single particle diffraction patterns, then SA will probably not give sufficient intensity from such small particles. You will probably have to use some form of convergent beam diffraction. Whether this is done in conventional imaging mode, or using a nanodiffraction mode is up to you and your microscope. Whatever, you need a focused probe with small spot size, sufficient intensity to see a diffraction pattern without roasting the particle, and a convergence angle low enough to separate the diffraction spots (you may need to change the C2 aperture). Then press diffraction. This is fiddly, but should work. The really tricky bit is if you then need to tilt the particle to a specific orientation. I can only say, be patient! And don't expect anything nice like Kikuchi bands to help you, it is too thin. Ian MacLaren <i.maclaren@physics.gla.ac.uk> 20 Dec 2004

You also need to keep in mind that with SA diffraction, spherical aberration limits how precisely you can define the area from which you get diffraction. The error is of the order of 500 nm. Typically, the smallest practical SA aperture will be ~5 μm which results in a selected area of ~<500 nm. Consequently, if you really push SA diffraction and succeed in getting a pattern, it is likely to be from a region different from where you think it is coming from. Larry Stoter <larry@cymru.freewire.co.uk> 20 Dec 2004

TEM - CdTe nanoparticles

I need some expert advice so I can help a user in my lab. He wants to see some nanoparticles he has synthesized by the following procedure: The sample is CdTe, a highly fluorescent NP with a shell of either thioglycolic acid or 2-mercaptoethylamine. In theory they should be about 2-5nm in diameter, but they are synthesized in aqueous solution, and in order to properly separate the particles I perform a ligand exchange and re-dissolve into organic solvents (i.e., toluene). I put this solution onto a carbon coated Formvar grid. I don't see much, some junk, but nothing like nanoparticles. Is there something there and I can't see it? Would I see it, if it were there? Would raw CdTe particles at 2 nm size have enough contrast to show up? Could the solution be so concentrated that it looks like a solid field rather than separate particles? The solution he gave me didn't really dry on the grid like I thought it would. How fast does toluene evaporate and could it mangle the Formvar? Jonathan Krupp <jmkrupp@cruzmail.ucsc.edu> 06 Jan 2005

I have had a few experiences looking at quantum dots and zeolite precursors for some of the materials scientists and chemical engineers here, and I may be able to answer some of your questions. First of all, these objects are small and inherently hard to see, so it would be easy to go to a relatively high magnification, scan across a grid square or two and not see much. I used cryofixation and looked at frozen-hydrated material, which is generally lower contrast than particles on thin carbon; however, the uniformity of the background could be better for ice than for a carbon coat, and this would render the particles more visible. CdTe certainly has more contrast than what I was looking at, so that is not the problem. It is very unlikely that the material is too concentrated to see. Especially since the evaporation of the toluene was not as expected, the particles are much more likely to aggregate than to form a uniform layer. If you have an oven or even a warm room, you might try drying the toluene at a somewhat elevated temperature. If the toluene mangled the Formvar, I'd expect to see prominent, irregular features (as happens when one gets poor Formvar removal using chloroform). If the particles as prepared are well-dispersed in the aqueous solution, and if you have access to cryopreparation equipment, you could try looking at plunge-frozen specimens. Measuring the fluorescence of the solution should allow you to calculate the number of particles per microliter, so you could determine roughly how many particles you would expect to see in a field at the magnification you use. Bill Tivol <tivol@caltech.edu> 06 Jan 2005

I know that individual 5 nm gold spheres start to become difficult to see in a real sample on carbon/Formvar grids. This is not so much size as the density you will get against a relatively thick background coating (>50 nm). Cd and Te are both lighter than Au and if the particles are not too symmetrical then I would have thought that they would be practically invisible. It sounds like one of those classical EM problems where the resolution of the microscope is not the issue but the resolution and contrast of the sample may be. I suppose you could play around with the voltage and aperture size to enhance contrast a bit, as well. Someone has already mentioned shadowing but I wonder if a simple negative stain might help–I really have no idea. But I'd be interested to hear how you get on. It would certainly be worth trying with the bare particles if you can. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 06 Jan 2005

SEM or TEM? For either, we just deposit the particles on Formvar coated grids, with or without carbon, directly from the water solution–just as long as there are no salts or the like to precipitate. This works with colloidal particles down to 3 - 5 nm, and composed of Au, Pt, Pd, Ag, Fe, and combinations and alloys of these. The particles separate fine without doing anything else. This is also true for particles conjugated to proteins like antibodies. I did try to look at some homemade Cd/Se quantum dots that had been synthesized in toluene (and a couple of other organic solvents, but I forget which), and it was a no-go. The toluene attacked and either dissolved or "wadded up" the Formvar film. This may be what happened to your samples. Philip Oshel <peoshel@wisc.edu> 06 Jan 2005

I think Formvar support film is not good for this type of experiment. You need a thin carbon film to reduce scatter from background and enhance signal-to-noise ratio. I had difficulties seeing nanogold particles (about 2 nm in diameter) in bright but not dark field. In dark field on 1.8 nm carbon they are perfectly visible. Your particles have lower density, so it would be even trickier to see them than gold. Sometimes it is easier to see the particles on the film rather than the screen (so you could focus on some junk in hopes of having your object nearby). I think it's not so easy to see nanosize objects on the support film. Negative staining would just complicate the situation because uranyl acetate staining, for instance, generates approximately 0.8 nm granularity of background. I don't think you could resolve 2 nm objects well with a 0.8 nm probe. Sergey Ryazantsev <sryazant@ucla.edu> 06 Jan 2005

It is quite difficult to view the 2 nm particles, besides being very tiny and thin they will have a very little contrast and it further depends on the atomic weight. We have successfully viewed 2-3 nm CdS particles on Formvar film. You have to play with the aperture, accelerating voltages and spot size. At 200 kV you will get better resolution but I mainly use an accelerating voltage of 120 kV, which gives better contrast. Moreover, the alignment must be perfect. We align the gun every time and correct stigmation for such samples. One needs absolute concentration and patience. Aarti Harle <aarti_harle@yahoo.co.in> 07 Jan 2005

We have successfully imaged 1 nm particles under even more adverse conditions. That is, in 70-nm thick sections of epoxy resin. One has to collect two digital images and subtract them. The one is run through a 3x3 kernel to smooth it, and the 1-nm gold image drops out of that one. I don't know whether you have digital image collection and processing, but if you have, this would be an easy way to solve your problem. Carol Heckman <heckman@bgnet.bgsu.edu> 07 Jan 2005

TEM – Carbon Grids

I am a graduate student trying to make my own carbon coated grids. I have no problem depositing a carbon film on a glass slide and floating the film on water. I then drop the grids on the film. My problem occurs when I swipe the grids off the surface of the water with a glass slide. The residual water on the slide wrinkles and tears the film, so I am left with an uneven, broken film across the surface of the grid. Does anyone have advice on how to get the grids out of the water without tearing the film? What about grid glue? Is this required? Steven Dublin <sdublin@emory.edu> 27 Jan 2005

Instead of picking up the carbon from the water surface with a glass slide all at once, it may be possible to break up the carbon on the water surface slightly and pick up a small 3 mm piece individually with each grid from underneath. Usually for carbon-only grids for virus examination, we would evaporate carbon onto freshly cleaved mica pieces (available from EM suppliers). We then floated the carbon films onto the surface of the water. Using freshly acetone-cleaned grids, we put one grid at a time underneath the pieces of carbon and pick it up. Freshly cleaning them would lower the surface tension when the grid went into the water. We generally used 400 mesh Cu grids to support the films. I have used grid glue routinely for picking up sections on naked grids but have never found it necessary for carbon only grids. Judy Murphy <murphyjudy@comcast.net> 27 Jan 2005

Try to use piece of Parafilm, instead of glass, to pick up the grids. Parafilm should be larger than carbon film. You need to completely immerse the Parafilm film into the water and than remove it quickly. Grids should be clean and stick to the carbon. Depending on carbon thickness, you need to use grids of corresponding mesh. Carbon which is slightly brownish

(so you could see carbon by naked eye) may be mounted on 400-mesh grids. The alternative way is to put plastic film on the grids first, then evaporate carbon and then dissolve the plastic; it would "glue" the carbon to the grid. You may also leave the plastic on the grid. Generally, carbon does not stick well to the naked grids, so people have used plastic support films or "holey films". You may also "pre-treat" grids with plastic (a drop of very dilute plastic solution per grid, dry before use) before mounting the carbon. Sergey Ryazantsev <sryazant@ucla.edu> 27 Jan 2005

It has been a long time since I've made carbon coated grids, but one of the approaches we used was to wrap a piece of coarse copper mesh around some wire (coat hanger would do) to create a support that we could place under water. We then dunked the grids through the water onto this support, and then floated off the carbon film onto the surface of the water. We were then able to manipulate the grid holder under the carbon film, and pick up the whole thing through the water. The simple method, of course, was to make thin Formvar coated grids, and evaporate some carbon on to them. Does your work preclude this approach? Joel Sheffield <jbs@temple.edu> 27 Jan 2005

Microtomy - Sectioning Woody Plant Stems

I have a researcher who would like to create cross sections of 2 - 3 year old pine sapling stems. The sections do not need to be terribly thin (50 - 100 µm) but he would like to have the surface as smooth as possible without embedding the tissue. Richard Harris <rjharris@uwo.ca> 28 Jan 2005

If you have access to a Vibratome then use that to cut the sections. Also, with some practice you may be able to cut free-hand sections that are thin enough for your purposes. Alternatively, you can buy little hand microtomes from major science or school supply companies. These cost about $100 and have a sample chuck that is raised by a little micrometer screw assembly through a flat platform. You insert your stem and then gradually raise it in height. Then you slide a long razor blade across the piece using the platform as a support. It is really a simple but very useful device. If we have a tissue that is too soft or small to hold well in the chuck, we just hollow out a piece of carrot and place the twig, etc inside. This will clamp well and hold the tissue well to get minimal crushing and distortion during cutting. Debby Sherman <dsherman@purdue.edu> 28 Jan 2005

I would recommend a sliding microtome (aka sledge microtome) if you can find one. The other possibilities are a cryostat or hand sections made with a double-edged razor blade. The hand sections are OK if he doesn't need consistency. Kim Rensing <krensing@interchange.ubc.ca> 29 Jan 2005

A good sharp wood plane iron or wide wood chisel, sharpened as you would a microtome knife, are much better than the straight razor that usually comes with them. Cementing a glass microscope slide on each side of the hole in the face of the microtome will help too. Trim the glass slides so they don't cause a danger to you the operator and use cutting edges made from good steel not the imported stuff. You may have to find an old chisel or plane to get a good piece of steel but the good modern brands should be OK if you don't have and old one laying around. Razor blades and straight razors are not stiff enough to make good sections from soft tissue let alone hard stuff. Try cutting with a slicing motion instead of with the blade at 90 degrees to the direction of travel. Gordon Couger <gcc@couger.com> 29 Jan 2005

Having cut a lot of wood and especially pine, maybe I can add a comment or two here. The requirement "smooth as possible" is a little vague; it would help to know how these sections are going to be examined i.e. optical, SEM, AFM? 1. From my experience, I've found the best way to section this material is using a regular microtome knife; disposable microtome blades just don't hold up well enough. A good well sharpened microtome knife will do a great job and stand up to the hard xylem elements. 2. Depending on the diameter of the sapling stems, they may have to be surrounded (not embedded) with something to make them more rigid. Options range from Paraplast to epoxy depending on how hard the stem material is. 3. When sectioning, it helps to soak the block in ice water (or warm water depending on the material used in #2) to soften the woody portions of the stem. 4. Fixing Pinus tissue is a whole other problem! Damian Neuberger <neuberger1234@comcast.net> 29 Jan 2005

If your researcher wants to use fresh wood sections, the sliding or sledge microtome suggested by Kim Rensing will do a great job, as long as your knives are sharp. We found that the disposable blades are not stiff enough for doing this on the sledge microtome, you need to use the large metal blades. A colleague recently sectioned 2-year old poplar stems this way. We're lucky that a local histologist will sharpen the blades for us. If you use a hand microtome, a non-flexible blade, something like a cut-throat razor, is probably necessary to get reasonably consistent sections. With hard tissue, the flexible blades chatter on the sledge microtome (on our one, anyway), and tend to flex and scoop out the middle of the tissue on a hand microtome. For support material, we use high density foam, the type used for insulating refrigerators. We have a large slab of this and just cut a small piece approximately to shape to fit around the tissue, in the same way we get students to shape carrot pieces around tissue for hand sectioning. Everything from shoot apical meristems to cores of 50-year old trees have been sectioned this way on the sledge microtome. Rosemary White <rosemary.white@csiro.au> 30 Jan 2005

The following worthy NetNote Items were excluded from the January issue of Microscopy Today for lack of room. They were posted on the microscopy listserver in late 2004 ...Editor

LM - Use of LM Stains on Plastic Embedded Plant Tissue

I have a collaborator who is interested in looking at some plant material using the LM. He is interested in the staining of lignin and pectin using conventional techniques (staining these

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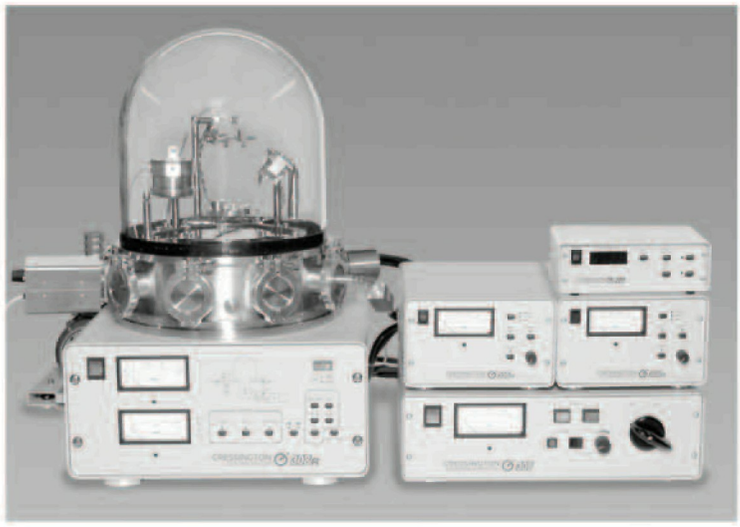

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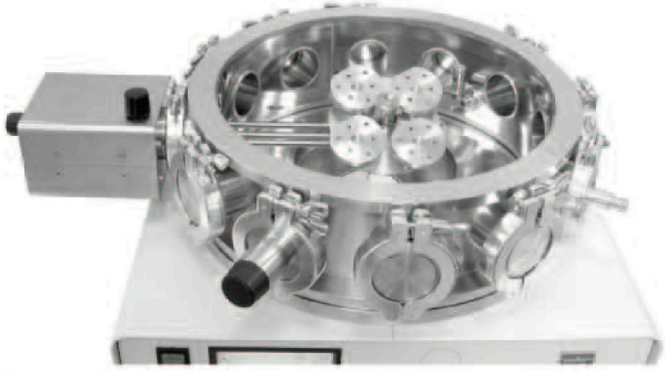

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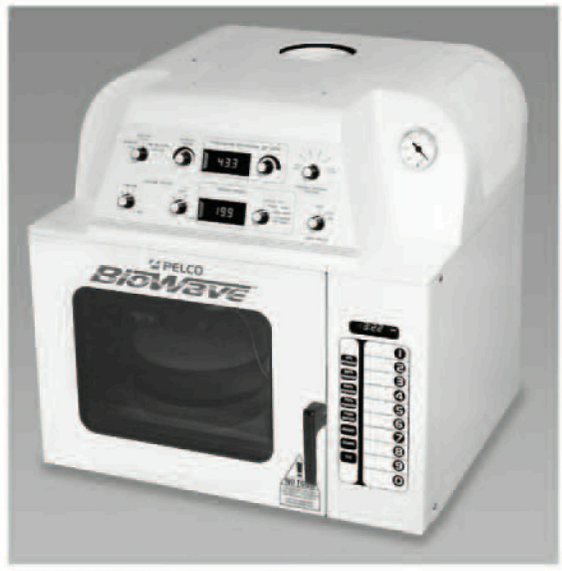

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with colored stains where the components would stain different colors and could be distinguished from each other). My background is EM, and I am not set up for paraffin embedding, so it will have to be some sort of plastic embedding. I know that there has been some discussion before on the use of LM stains on plastic sections. I'm wondering if the stains (such as Triarch quadruple stain) normally used for lignin and pectin and other plant materials would work if the plastic sections were etched or the plastic removed altogether or if there are other stains which I could use. I hope to do some immuno work on these samples eventually, and maybe some TEM, but the LM has to be done first for us to decide whether to do the other techniques. My questions are: 1. Can the specific botanical stains be used on plastic sections 2. Is there a preferred embedding plastic which I could use (LR White, methacrylate, others?) Right now the samples are sitting in 2.5% glutaraldehyde in 0.05 M cacodylate buffer. Paula Allan-Wojtas <allanwojtasp@agr.gc.ca> 21 Oct 2004

Try Unicryl. It is a reliable dual-use EM/LM resin. LR White may work for you too. Chris Jeffree <c.jeffree@ed.ac.uk> 21 Oct 2004

Generally, stains that 'work' in paraffin also work in plastic. There is not even any need to etch. The stains are small molecules and they penetrate easily. To get information about specific stains, check the book by Steve Ruzin called Plant Microtechnique and Microscopy, or Botanical Microtechnique and Cytochemisty by Berlyn and Miksche. Or if you can find it the book by O'Brian and McCully. Having said that, if contrast is the only issue, its fine. But if you really want to know that the color is pectin, it is probably better to use antibody staining. There are quite a few good ones of those available. I think the dye stains for lignin are reliable, and I don't know of antibodies for that. For plant material and immuno, I have had very nice results with butyl-methyl-methacrylate, and I'd be happy to send you a protocol if you like (mainly for light level work). Tobias Baskin <baskin@bio.umass.edu> 21 Oct 2004

LM - Mounting Medium

I would like to know what mounting medium is used for Araldite or Epon semi-thick sections (2-5 micrometer). We have used Canadian balsam so far but this causes wrinkles of sections under the cover glasses. Kärt Padari <kartp@ut.ee> 08 Nov 2004

Xylene-based mounting media often cause wrinkles in 'thick' sections. Lots of labs just use a drop of the same embedding medium (with accelerator) the section is in (Epon, Araldite or Spurr). Geoff McAuliffe <mcauliff@umdnj.edu> 08 Nov 2004

Why not use the same resin? We do. Put some left-over resin from an embedding run into a 1 ml syringe. This is ideal for controlled dropwise delivery. You can seal with Parafilm and keep a stock syringe or two in the freezer for future use. Chris Jeffree <c.jeffree@ed.ac.uk> 08 Nov 2004

I simply use Permount. Peggy Sherwood <msherwood@partners.org> 08 Nov 2004

We have used immersion oil sealed with nail polish and it has worked very nice. Robert Underwood <underwoo@u.washington.edu> 08 Nov 2004

TEM – Diamond Film

Can any one tell me how to thin a diamond film or cross section the diamond film for HRTEM? <rangari0@yahoo.com> 13 Oct 2004

You should use diamond abrasive paper to ground the sample to about 40microns, then ion-mill the sample to electron transparent! Changhui LEI <clei@uiuc.edu> 13 Oct 2004

You don't mention your substrate. I assume that this is a diamond film on a substrate such as silicon. If your film is on a Si substrate, then I highly recommend the small angle cleavage technique or Microcleave technique. Check the South Bay Technology website for their microcleave kit and associated PDF files by John McCaffrey and myself. If you must do ion milling, then you should be aware that carbon films do not ion mill very well with Ar beams. However, if you have a low angle mill, then things are alleviated quite a lot. One of the things that people have done to overcome the low milling rate of carbon is to add a little O2 into the Ar. Try 20%.) This is essentially a reactive etch for you. I have a paper in the MRS number four series on TEM sample prep that shows the relative differences of ion milling rates with Ar, Ne, Ar+ O2, and Ne+ O2 gases in a Gatan Duomill at 10 degrees. We used AFM to measure the relative heights of a DLC film on a TiC film on a silicon substrate. The idea was to try to match the mass of both the C and Si with the gas as close as possible. The best results were obtained using Ne+ O2. Ne did a good job also. Scott D. Walck <walck@ppg.com> 13 Oct 2004

TEM - *Haloferax Volcanii*

An investigator in my department wishes to have me do TEM on Haloferax volcanii. I have never worked with an organism that has a 3 M internal salt concentration. Has anyone worked with these? Pat Connelly <psconnel@sas.upenn.edu> 01 Nov 2004

The crucial point in the TEM processing of Haloferax (or any other extremophil for that matter) is a very gradual desalting and pH increase. After the initial aldehyde protein crosslinking (we use 2.5% glutaraldehyde made up in the growth medium), the cells should be brought to the neutral pH before the dehydration. The rule of thumb is that the previous solution shouldn't be more than 10x more acidic than the current wash (~factor of 1). A good indicator of going too fast is a dramatic pellet size decrease - as the cell membranes rupture due to the osmotic imbalance. Alice Dohnalkova. <alice.dohnalkova@pnl.gov> 01 Nov 2004

SPUTTERING - Thickness of Sputtered Gold

Could anyone please tell me a simple way to estimate the thickness of sputtered gold, for example by light or IR absorbance of a film on glass? Robert H. Olley <hinmeigeng@hotmail.com> 24 Nov 2004

A simple method is to use a color spectrophotometer in a transmission or reflectance mode and use thin film modeling techniques. See any book on spectrophotometry of thin films or ellipsometry. There are commercial thin film programs available. Two that I am familiar with are TFCalc and FilmStar. You can also write your own. The principles are straightforward and you can implement them even in Excel. The optical properties of gold, n and k, as a function of wavelength are well known and can easily be found. You might want to try to get your hands on some back issues of Vacuum Technology and Coatings. Peter Martin from Pacific Northwest National Laboratories has a regular series in it and he had one on optical coatings and how they are characterized. The basics were covered in his articles. Scott D. Walck <walck@ppg.com> 24 Nov 2004

We used AFM to measure the thickness of sputtered coatings. Evgenia Pekarskaya <pekarskaya@mail.pse.umass.edu> 24 Nov 2004

EBSD - Insulating Material

I have a glass-ceramic material, where glass and crystallites have the same composition. I want to perform the EBSD on crystallites of this material. I can not coat the material because I have to do diffraction. I used SE mode with 0.3 torr water pressure but the image quality is not good enough for 5 micron size crystallites which have the same composition as the glass and which are sticking out only few nanometer out of the glass surface (50-60 nm, I can make it 100-200 nm with etching but still having difficulty in finding out crystallites). I used the gas detector with 1 torr pressure and image quality improved but the diffraction pattern quality degraded to a level that I can not map it properly. Is there a simple way to do EBSD of these materials? Pradyumna Gupta <png2@lehigh.edu> 20 Oct 2004

When doing EBSD with non-conductive small geologic particulates I've actually had the best results using an extremely thin carbon coat and high vacuum rather than low vacuum. Never having measured it, my best guess is the coating is 5- 10 nm - or as absolutely thin as you can get it, using a good vacuum (10-7 mbar range). Richard Edelmann <edelmare@MUOhio.edu> 20 Oct 2004

I would not etch the material at all; EBSD requires a very flat surface. The best is to go down all the steps of grinding and polishing to one micron diamond, then long polishing with 0.05 micron silica colloid (Syton). You may well find that the conditions for good EBSD are the opposite of the conditions for good imaging; you just change the conditions for each one. You will find that BSE imaging is best and works at any pressure. A colleague who did minerals said he polished, then did a very thin carbon coat, then polished again, then coated again, etc. This filled in any tiny cracks. A very thin carbon coat will not harm x-ray diffraction studies; it is invisible to the x-rays. Mary Mager <mager@interchange.ubc.ca> 21 Oct 2004

EMBEDDING - Epon Sections Diffused

Somebody in our lab has had sections that will get diffused and dispersed within two seconds of staying in the boat. All the Epon ingredients were brand new. Any suggestions what's wrong or can be done about it? Fanny Chu < fchu@mrl.ubc.ca> 08 Dec 2004

We've all been hit with this phenomenon at some time. It sounds like incomplete infiltration or polymerization to me. Even with brand new components, if they are not mixed adequately, the resin won't harden correctly. Also, if the dehydration was not complete (your absolute ethanol or acetone was not actually absolute) the resin will not be able to fully infiltrate the tissue and you will see the effect you've described. I'm afraid there is no salvaging of those blocks. Next time, make sure that you use a fresh bottle of 100% dehydrating agent, and also take the time to thoroughly mix the resin components. It may even help to stir them up, let them sit for 15-30 minutes and stir them again. We used to do that with Spurr's resin all the time. Leona Cohen-Gould <lcgould@med.cornell.edu> 08 Dec 2004

When I prepare my Epon resin I stir two components together for 5-10 min, add the third, stir for another 5-10 min before adding the accelerator and stir for 30 min. I get consistent result this way. If you put all four together and stir, you may get various results. Ann Fook Yang <yanga@agr.gc.ca> 09 Dec 2004