NETNOTES

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Selected postings from the Microscopy Listserver (http://microscopy.com) from 4/14/06 to 6/14/06. Postings may have been edited to conserve space or for clarity.

SAMPLE PREPARATION - Critical point drier valves

I'm using an older critical point drier. It has these knurled steel knobs for opening/closing the valves. Controlling the valves kills your fingers since the rough metal is hard on your skin. Anyone modified it with plastic knobs or rubber covers or something to make it less of a literal pain to do a critical point drying run? <gvrdolja@nature.berkeley.edu> 10 May 2006

Two simple tricks to keep your skin more or less intact: - I never adjust the coarse vent valve. Permanently leave it halfway open and control venting with the needle valve. - When going through the fill-drain cycles — open the fill valve one-half turn or so and leave it there until you're finished. Then you just open the drain valve to let fluid out, and close it to let fluid in. This cuts the number of knob-turning operations down by half. On our unit, the fill valve is the hardest to operate so this trick is very helpful. I'd avoid pliers unless you absolutely can't get the knobs to turn. Pliers can strip the knurled texture and just make things harder in the future. And too much force can damage the valves. I do have one user that uses vise-grip pliers on the fill valve, but she is under stern warnings to pad the knob with cloth and not use force to close the valve. Rick <hugo@pdx.edu> 11 May 2006

All this talk of forcing needle valves and pliers, etc. on critical point drier systems is scaring me. There used to be a homemade CPD in the Berkeley Microlab that sounds very similar to the unit currently being discussed. It was retired chiefly because of safety concerns. The pressures and explosive-release volumes on these systems are sufficient to cause serious injury in the event of a failed valve or fitting. Trust me — It's not worth it. If you find you are using hand tools to adjust needle valves on these systems, you do have a safety problem. Mike Young <mike.young@yale.edu> 11 May 2006

The first question I would ask: Why are the knobs hard to turn? Generally it's a couple reasons. 1) If the threads are lubricated and the grease/oil and enough of the VOCs (even if they aren't very volatile) dissipate, the lubricant can turn to glue. Solution: Disassemble, clean (with WD-40, CRC or other solvating lubricants), and re-apply some lightweight grease (lithium or wheel bearing would be fine), or a touch of heavy oil. 2) If the threads/valve are damaged, you probably should replace the valve. These all are extremely simple devices. And as such, parts are relatively easy to repair/replace or fix. Assuming you have the patience to source the proper valves. The pressures on the CPDs the microscopy community utilizes are relatively insignificant to some of the devices folks in physics and engineering play with daily, not to mention, McMaster-Carr has a great selection of valves and what not that are rated well above the typical 1500-3000 psi burst limit (safety) on the CPDs. If the valve was always hard to turn that's one thing; if it is now, make it easy to turn. Try even spraying the threads with some WD-40 or Liquid Wrench and run a cycle or two and see if that helps anything. Geoff Williams <geoffrey_williams@brown.edu> 11 May 2006

SAMPLE PREPARATION – negative staining enterococci

We have problems with negative staining of E. coli, and have tried two different approaches with both uranyl acetate and PTA. Without being able to detect the flagella, we see partly collapsed bacteria and lots of dirt (growing bacteria directly on the grids). We've also tried to embed the E. coli in a mixture of methylcellulose and uranyl acetate without any luck. We've used carbon coated Formvar films on copper grids. I enclose the procedure for preparing cells grown on grids, observations and some questions form the scientist in charge of this project. We might be wrong trying to grow cells directly on grids? Our aim is to prepare grids for TEM with a suitable cell number for reliable analysis without damaging the cells by centrifugation. We plan to grow the cells directly onto the grids and have performed the following: Inoculate cultures of enterococci in TSB + 0.25% glucose and grow overnight at 37°C without shaking. TSB is a bacterial growth medium made from casein and soya peptone. Dilute cultures 100-fold in 10 ml growth medium in microtiter plate wells containing TEM grids (should contain ~107 cells/ml). Incubate at 37°C without shaking for 1-4 hours. Pick up grids at different time-points (i.e. 1, 2, 3 and 4 hr) from the culture. Dip the grid in particle-free water. Stain the cells with 0.5% uranyl acetate for 30 seconds. Rinse the grids gently in particle-free water before air-drying them for 10-15 minutes. Observations made: 1. there are low cell-numbers on the grids 2. there is a lot of stained debris or precipitate on the grids and it seems to increase during the incubation. Questions: 1. What cell density is required to obtain one layer of cells on the grid but not cells on top of each other? 2. Are there ways of increasing the bacterial affinity for the grids? 3. Are there ways to reduce the amount of debris on the grids? 4. Can grids be incubated on top of a nitrocellulose filter without being damaged? Randi Olsen <randi.olsen@fagmed.uit.no> 19 May 2006

I must admit that I have never attempted growing bacteria on grids. But I wonder if the copper of the grids is too reactive. It might both inhibit bacteria and encourage precipitation/reaction products. You could try a couple of gold grids as a control to see if matters improve. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 19 May 2006

A possible way out of your problem might be the technique I used years ago in the study of thermophilic bacteria in nature. I deposited carbon films in vacuo on small freshly-cleaved mica squares and let them mature for a few days. The films were carefully floated off on the water surface and the bacteria were allowed to settle on and attach to the underside of the carbon. The films were then transferred to other solutions with a fine Pt loop (or even the original mica piece - submerged then lifted up below the film). Staining with aqueous solutions of UA, AM or PTA can be done either before (preferably) or after the films are picked up with TEM grids. Jim <jchalcro@neuro.mpg.de> 19 May 2006

There are two potential problems with the approach you describe. One is that the copper grids, as pointed out in another reply, may be reacting to the salts in the growth medium. The other problem is that the bacteria are in the process of forming a biofilm on the support film surface during the incubation time. If you only want to examine the bacteria by negative stain, you can place a drop

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of the culture on the support film surface, leave for about 30 sec and then replace the drop with a drop of negative stain. You have to be careful to make sure there are no salts in the growth medium that could cause the negative stain, and in particular the uranyl acetate, to precipitate. There is no need to centrifuge the bacteria because there are usually enough in the drop to fall to the surface. As with most techniques, there are many variations on this simple method, but it is always a good policy to try the easier way first to make sure it is possible to obtain results. The next easiest way is to deposit a carbon film onto a freshly-cleaved mica surface and then allow a drop of bacterial suspension to infiltrate between the carbon film and the mica. If you then place a clean grid on the carbon film you should be able to pick it up onto the support grid and then negative stain. The advantage of this method is that you don't have to worry about the support film being hydrophilic - it already is. The disadvantage is that the carbon film is very fragile so it needs a small mesh grid to support it. Even better support comes from holey grids. If you are interested in examining the bacteria as they grow on the grids, first switch to gold or nickel grids. You may then want to extensively wash the bacteria before you stain them to remove all the extracellular material they secrete in the short time they have been incubated. You can check to see if what I am saying is correct by taking a grid with bacteria on directly from the culture medium (after incubation) and plunge-freezing it in liquid propane. From there you freeze substitute it in ethanol (with a little osmium tetroxide if you wish), warm it slowly to 4 degrees and then critical point dry it and look at it in the SEM. My guess is that the bacteria will be almost invisible because they will be covered in a large amount of slimy looking stuff. I hope this helps. Paul Webster <pwebster@hei.org> 19 May 2006

I do not have answers for all the questions but this is what we have done in the past while negatively staining Gram negative bacteria. It might be challenging to achieve a monolayer of bacterial cells on the grid as every bacterium behaves a little differently when it comes to attaching to any surface. In order to view flagella or pili, we grew the bacteria in stationary cultures to prevent mechanical loss of flagella or pili. Once the bacteria were in their log phase we pipetted out around 100 microliters of the culture and floated a Formvar coated nickel grid on it for about 5 min and then let the grids dry. We found the incubation time to vary with different strains. These grids were rinsed in distilled water for 30 sec to a min. I have found that rinsing the grids was very important to remove all the debris and residual media from the grid leaving a clean prep. If you find low number of bacterial cells you can try increasing the incubation time and decreasing the rinse time. Staining: The grids were stained for 10 min with 0.2% uranyl acetate for 10 min followed by a 1 min rinse with distilled water. The grids were then air dried before examination. I found rinsing steps were keys to a good preparation. You have to play with the inoculation and rinsing steps to obtain desirable results. Vinod Nair <nairvinods@gmail.com> 22 May 2006

SAMPLE PREPARATION – dispersing powder samples

I need to make samples dispersed on carbon-coated grids for collecting some EELS standards of various oxides in powder form. I have Manganese, Titanium, and Ruthenium oxides in various states. Anyone know a good prep that will evenly disperse a thin layer onto a carbon-coated grid? My first thought would be to mix with a solvent and put a drop on a grid, but I figured someone here has probably already done this. Leslie Krupp (Thompson) <lkrupp@us.ibm.com> 01 Jun 2006

My choice would be your first thought. It worked very well for river-bottom sediment. I would try various dilutions to find the one that gives the desired distribution of particles, and I would glow-discharge the grids. If using water leads to aggregation, try an organic solvent, and if the particles still have a tendency to aggregate, try putting the grid with the drop of solvent in an oven; faster evaporation might overcome that tendency. Bill Tivol <tivol@caltech.edu> 01 Jun 2006

We too have used the method you've described, but I am not familiar with "glow discharge the grids". What is it and how does it work? Lou Ross <rosslm@missouri.edu> 01 Jun 2006

Essentially, glow discharge is a procedure that you can carry out on a standard vacuum evaporator that is equipped with a high voltage AC input. Unfortunately, not many vacuum evaporators have this accessory. In operation, specimens are loaded into the chamber and one end of about 8-12 inches of high purity aluminum wire (3-5 mm) is plugged into the HV feedthrough and a large loop is formed to surround the grids. The chamber is pumped down using only the rotary pump until you get approximately 50-100 millitorr vacuum. At this point, you turn up the AC high voltage (using a variAC) and you will generate a plasma similar to what one sees in a sputter coater. The plasma is thought to both clean the grid surface as well as imparting a charge that renders the grid surface (of carbon-coated grids) hydrophilic. The effect lasts about a day, less if in a high humidity environment. We have been able to implement this technique by placing the grids inside a sputter coater and shielding them from direct line of sight with the target (to prevent coating with metal). The plasma generated will impart a hydrophobic character to the grids. But you may also get metal deposition, so do some trial runs first. I have been depositing a lot of nanocrystals as you describe by directly depositing them on carbon or silicon substrates. The powders are suspended in acetone, shaken and (after allowing the large particles to settle) a small volume taken up in a micropipetter (about 10-20 microliters). This is then dropped directly onto the grid surface from a distance of 10 or so mm. John Bozzola <bozzola@siu.edu>

SAMPLE PREPARATION - metallic glasses

Is there anybody who could give me a hint concerning the acid or acid mixture that would be effective in surface etching a piece of ternary metallic glass having the composition $Zr_{70}Ni_{10}Pd_{20}$? I need to reveal the dimensions of grains which are most probably formed in the material after a short annealing during which an icosahedral quasi-crystalline phase was formed (it was revealed by X-ray diffraction). Any suggestion will be very welcome. Corneliu Sarbu <crnl_srbu@yahoo.com> 09 May 2006

Considering Ti as chemically similar to Zr, I found some etchant solutions for Ti alloys in Metals Handbook from ASM. That these might work for Zr alloys is supported by comments in Cotton and Wilkinson, Adv. Inorg. Chem. Anyway, here are a couple that ASM suggests as general purpose etchants: 10 ml HF, 5 ml HNO_3, 85ml water 1-3 ml HF, 2-6 ml HNO_3, water to 1000 ml You can also try contacting ATI Wah Chang in Oregon, USA.

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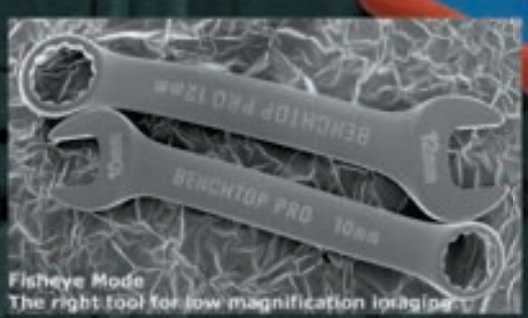

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They've been producing Zr alloys for decades and have plenty of expertise in that area. Be careful if you use HF. Rob Bowen <rob.bowen@caddock.com> 09 May 2006

SAMPLE PREPARATION - Time course experiment

I have recently had a discussion with a colleague about the best protocol to follow when staining cells during a time course experiment. I don't think there is a single correct answer, however, and would like to know current thinking on the following issue: Live cells were treated with a compound and observed at various time points during a period of 48 hours. At each time point, cells were fixed and immunofluorescently stained for the protein of interest. Is it a less artifactual procedure to fix cells at each time point and keep in a buffer until the end of 48 hours to stain them all at the same time or fix and stain at each sampling time point? To stain at the same time may reduce staining differences; however, keeping cells in buffer for different times may induce changes in the protein. I look forward to hearing your opinions. Judy Trogadis <trogadisj@smh.toronto.on.ca> 02 May 2006

An end run around the problem is to start off the treatments at different times so they all end together and then fix and stain is all done at the same time. This doesn't answer your question but maybe a useful wrinkle? Tobias Baskin <baskin@bio.umass.edu> 02 May 2006

There are some other things to consider. First off, a great deal of this debate will depend on what you are looking for and how it reacts with your fixative. If the cells are 'lightly fixed' there may be some reversal of fixation with prolonged buffer storage. Does that effect the staining? Tobias offered a good suggestion but there might be some chrono effects, cells fixed at different times of the day or night depending on your experimental design. I suggest avoiding all problems and debate by keeping all of the fixation, buffer wash times and staining times the same. Your staining procedure should be sufficiently standardized so that it is not a variable, or is the least problematic of the potential variables. Finally, people looking for something to criticize in your procedures will always find something 'wrong'. Geoff McAuliff <mcauliff@umdnj.edu> 02 May 2006

I don't know in detail what you want to observe, but isn't there a fluorescent tracker-molecule (such as a Lyso-tracker) available for your purpose? Another more ideal solution might be creating, for that one protein, a GFP-positive cell-line! Then you could make a continuous time-lapse without the need of fixation etc., all depending on your experiment's requests of course! Anyway, if the fixation is strong enough, does the protein still show activity / are changes still induced? To my opinion and experience, if fixed strong enough and there are no/little changes, it should not matter whether the cells are stained immediately or a few hours later, especially when also stored cold. Sven Terclavers <sven.terclavers@med.kuleuven.be> 02 May 2006

SAMPLE PREPARATION – Formvar film problems

Due to their price and very long delays of delivery, we decided to make our Formvar grids ourselves. But we have a problem: the film is full of holes! These are small holes about 50-100 nm in diameter, without sharp edges. I wondered if it did not come from traces of fine water droplets that remained on the glass slides. Here is my protocol: I clean a glass slide by breathing on it and then rubbing with a dust-free

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towel. If I clean the slides with alcohol, I noticed that the film sticks to the slide and does not detach in water. Then I let the slide dry under the hood for 5 min. Then I plunge the slide in Formvar (10 sec) and take it out. I let it dry in the hood for 5 min and cut the side with a razor blade. Then the usual plunge-it-in-water-praying-that-the-film-nicely-detaches-without-making-problem-thank-you-God. Any suggestion? Stephane Nizet <nizets2@yahoo.com> 27 Apr 2006

Holes are due to water in your Formvar solution. A very low water content causes these holes. I presume you dissolved your Formvar in water-free chloroform? Did you dry (e.g. in a Speed-Vac) your (ethanol) wetted Formvar slug before dissolving it? Peter Heimann <peter.heimann@uni-bielefeld.de> 27 Apr 2006

I use much the same technique, so I would suspect moisture absorbed by the chloroform/other solvent. If you're using an old bottle, poor quality or shared solvent, it might be worth trying a fresh bottle of reasonable reagent quality and keep the lid on all the containers as much as possible. For instance, I pour my Formvar into a measuring cylinder in the fume hood and dip my slides in it one at a time. But in between dipping I have a small glass beaker that snugly fits over the neck of the measuring cylinder as a lid to reduce the risk of moisture getting into the Formvar solution. I change the Formvar solution when it starts to make films with too many holes. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 27 Apr 2006

I usually clean my slides with acetone just before dipping them into the Formvar solution. Spray each side with a spray bottle, wipe off (once) with a lint free tissue and make sure there is no acetone left on the slides. After taking the slide out of the solution I hold it in the vapor of the Formvar solution for ~30 sec to make the film slightly thinner and more even. And I huff on each side of the slide after I have cut the film, to make it come off easier in the water. Plus, something I realized in Australia, if it's a rainy or humid day, your film is much more likely to have holes in it. In fact, it was almost impossible to make good film on rainy days despite air conditioning. Try a fine dry day for it but then the flip side is, you have to battle the dust. Cornelia Muncke <c.muncke@liverpool.ac.uk> 27 Apr 2006

We use Formvar dissolved in ethylene dichloride (dichloroethane). If we make it ourselves, I always dry the Formvar powder in an oven before using it. Often, because I am lazy, I will buy 1% Formvar solution from the EM supply companies. Either works fine. We have no problems with holes, but we are in a humidity controlled lab and we do keep the Formvar covered as much as possible, more to prevent evaporation of the dichloroethane than concerns about water. If you are not humidity controlled then you really should wait for dry days to make films. They keep for months and years if kept dry in a desiccator. We wash the slides by spraying with some distilled water and the gently wiping with lens paper. Let air dry for a minute or two and put into the Formvar solution. We also use film casters (bottom flask container and thistle tube...Formvar is pumped using a hand bulb up into the thistle tube and held there while the slide is inserted. After a few minutes the Formvar is drained out and the slide is left dry in the vapor before removing.) The film caster gives a consistently even film with large areas of the thickness desired. We regulate thickness by timing submersion in Formvar and then drying time in the thistle tube. Note that ideal Formvar concentration may be different if you are using the dip method rather than the film caster. If you plan to make films regularly than I strongly recommend investing in the film caster. It's a one-time purchase and most of our users make their own films. It is convenient and much less expensive than purchasing coated grids. Debby Sherman <dsherman@purdue.edu> 27 Apr 2006

There seems to be a general agreement that it is probably water contamination. This is comforting if I know the cause of the problem, but I still have to find a solution to it because: - I bought the Formvar solution ready to use in dichloroethane - I use it under the hood (of course) and close it between the slide (I prepare 2 slides at a time; we are working with air conditioning, at controlled 60% of humidity. - The weather was very nice these last days: very sunny and 24°C. Stephane Nizet <nizets2@yahoo.com> 27 Apr 2006

One possibility that hasn't been mentioned is that if the Formvar solution is colder than ambient, then when you pull the slide out of the solution some water vapor will condense on it. This is especially true on humid days. Incidentally, chilling the Formvar solution prior to breathing on the newly-cast films will ensure a large number of holes. Andy Bowling <abowling@mail.utexas.edu> 27 Apr 2006

I had heaps of trouble with my Formvar for a while. I'm in Vancouver, so rain is a fact of life here, especially in the winter. After trying everything I could think of (only doing it on dry days, fume hoods, different dipping methods and containers, buying new Formvar solution) I finally made my own Formvar from our stock powder in the lab in dichloroethane and have never had a problem since. Now I make the solution myself, throw it out if it gets to be 6 months old, or if it has been opened more than 5 times. It is possible that this is a tad over zealous, but it works for me. Robin Elizabeth Young <youngre@interchange.ubc.ca> 27 Apr 2006

So far no one has mentioned how to get Formvar films off of alcohol-cleaned slides. Our method is to clean the slides with ethanol, then apply a thin film of oil to the slides by rubbing the sides of your nose with your fingers and transferring the oil from your skin to the slide. An alternative is to dissolve Apiezon L in petroleum ether and dip the slide in that solution. In any case, the trick is to get the slide controllably dirty. When removing the film we use warm water, and when using the Apiezon, we add 0.25 g of Alconox to 1 L of water. Bill Tivol <tivol@caltech.edu> 27 Apr 2006

SAMPLE PREPARATION – Solvents that do not affect Formvar

I have a client who needs to find an anhydrous solvent in which to disperse her powdery stuff (ferrous and silicon oxide smokes, I think) that will not take up water, will not affect refractence spectra, and will not eat the Formvar on grids. This is for TEM and, perhaps, EELS. Any ideas? Tina Carvalho <tina@pbrc.hawaii.edu>20 Apr 2006

If you want to use a clean solvent for inorganic specimens I would recommend using holey or ultra-thin carbon films rather than Formvar. While Formvar is the standard thin film for examining biological specimens, and is perfectly beam stable under wide beam illumination at lower to intermediate magnifications it is particularly unstable under convergent beam/high beam intensity conditions (such as are typically needed for core-loss EELS). The main advantage, however, is that carbon films are stable for a wide range of ultra-low water anhydrous solvents, my personal preference

is for high purity Ethyl Ether, mainly as it is extremely volatile and seems to produce very low/no contamination build up (another problem for EELS). I'm sure everyone on this list has a personal solvent preference however. Matthew Weyland <mw275@cornell.edu> 20 Apr 2006

Ethanol will not eat Formvar, and 95% should be OK as far as taking up water. If even that amount of water is not acceptable, I'd try n-butanol. A real chemist could tell you if 100% butanol takes up water. I have no idea whether either of these will affect refractence spectra, or whether these spectra are to be obtained on the suspensions of the particles or on the particles themselves after the solvent has dried. I also do not know whether alkanes dissolve Formvar, or, if you don't mind dealing with some nasty smells, whether some of the substituted aromatics, ones like toluene, xylene, or pyridine, might be better on Formvar than benzene would work. Bill Tivol <tivol@caltech.edu> 20 Apr 2006

SAMPLE PREPARATION - Gelatin as the embedding media

Does anyone have experience cutting fixed tissue/insect brains using gelatin as the embedding media? What should the strength (bloom #) be? What percentage? I plan on using my ultramicrotome and a glass knife at ambient temperature to make about 60-80 micron sections. I will eventually apply immunocytochemistry to these sections. Any advice or suggestions are greatly appreciated! Chip Dye <dyel@mail.nih.gov> 25 Apr 2006

I use a Vibratome instead of the ultramicrotome for this. The insect brains are fixed and then embedded in warm (40°) 15% gelatin (any type of gelatin should be useful). When the gelatin has set in the fridge, I cut a little block out of it and section it on the Vibratome. 50 microns for light microscopy, 70 microns for pre-embedding TEM immunocytochemistry. Gerd Leitinger <gerd.leitinger@meduni-graz.at> 26 Apr 2006

For animal tissue I prefer to use agar embedding. It holds sections better when processed, for example, for further flat embedding of immunocytochemistry. I also cut sections with Vibratome, yet I use a sapphire knife instead of the standard blade to get sections about 35-40 micron thick which I prefer for immunocytochemistry. Albina Mikhaylova <amich@ufl.edu> 26 Apr 200

SAMPLE PREPARATION - neutralizing glutaraldehyde

I fixed bovine serum albumin with glutaraldehyde in 10 mM phosphate buffer pH 7.2 and I need to neutralize the glutaraldehyde in the solution. I need a way to keep everything in solution while neutralizing the fixation process (aldehyde + amine groups). I cannot remove the bovine serum albumin from the solution, but I could remove the glutaraldehyde. I was thinking about increasing the pH. Do you think glutaraldehyde loses its fixating activity at pH 10? Any ideas? Stéphane Nizet <nizets2@yahoo.com> 25 Apr 2006

We sometimes have concerns about the cross-linking activity of glutaraldehyde when doing preparations of virus suspensions where we do not want artificial aggregation. Simply put, dogma states that because glutaraldehyde is a 5 carbon chain with highly reactive carboxyl groups at each end it is more likely to cross link different virions, or virions with cellular detritus, than formaldehyde, which has a single carbon and a single reactive aldehyde group. Normally I use glutaraldehyde at a concentration of 0.1% to stabilize reactions. Over the years I have not really seen appreciable clumping which could be associated with the fixative. But if you are doing an immunoprecipitation style IEM procedure you really don't want to take the chance of creating an artificial situation. To protect against this I neutralize the fixatives in the sample by addition of glycine. Note: lysine is frequently used. However, it has two reactive amine sites, so I avoid that because, technically, it may also contribute to cross linking. The final concentration of glycine we use in the preparation is 8 mM to neutralize 0.1% Glutaraldehyde, and 125 mM to neutralize 2% Paraformaldehyde. Perhaps a chemistry minded member of the list will be able to provide for a better way of neutralizing. Paul R. Hazelton <paul_hazelton@umanitoba.ca> 25 Apr 2006

Perhaps an alternative would be NH_4Cl (ammonium chloride), applied in a washing buffer, end concentration 50mM, application time: necessarily only seconds (personal comment by Prof. Roth) , but usually for tissue specimens (as used in TEM-specimen preparations) 20-30 [up to 4 hrs] min, followed by one to two additional washes in the respective, pure buffer solution [e.g. 4 °C, overnight]. Source: Roth J. et al. (1981) Enhancement of Structural Preservation and Low Temp. Immunocytochemical Staining in Low Temperature Embedded Pancreatic Tissue. J.Histochem.Cytochem. 29, 663-671. Wolfgang Muss <w.muss@salk.at> 25 Apr 2006

Would it be possible to add a concentrated solution of Tris (or even to dissolve crystalline Tris into the BSA solution) to bind any remaining reactive glutaraldehyde? This would raise the pH though. One could also use a concentrated solution of glycine or even lysine if one wanted an additional amino group to aid in the binding. If this concentrated amino acid solution were pH'ed before addition to your BSA experiment I don't think it would alter the pH much. George P. Leser <g-leser@northwestern.edu>

MICROTOMY – Diamond knife damage

We have a problem with blocks damaging the diamond knife. Cell cultures grown in flasks and processed without any glass cause us much damage. We don't use molecular sieves. We use Araldite and just the blocks of cells from the flasks cause the damage. Rina Jeger <jeger@bgu.ac.il> 23 May 2006

We had that problem and it turned out to be the microtome rather than the blocks. If bearings are worn out or advance motors are not working properly you can get enough instability in the cutting stroke to cause fine damage to the knife. Debby Sherman <dsherman@purdue.edu> 23 May 2006

I always request that tissue culture flasks/dishes are rinsed with a sterile PBS prior to growing any cell lines to remove any debris that is in there. I had problems like that and I rinsed the flask, spun the content and checked under light microscope. There was plenty of debris, probably of plastic origin. It might ease the problem but it will not eliminate it 100%. If it is really bad I'd use a glass knife. Dorota Wadowska <wadowska@upei.ca> 23 May 2006

I had this problem. The problem diminished when I switched from smashing osmium ampoules to dissolve the crystals to buying made up solutions. Dave Patton <david.patton@uwe.ac.uk> 23 May 2006

A simple trick to eliminate smashing osmium vials is to dip the sealed ampoule into liquid nitrogen. This releases the osmium crystals from the glass walls. Then simply break the vial using an

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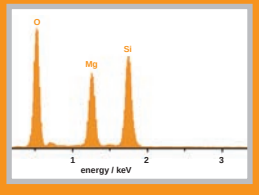
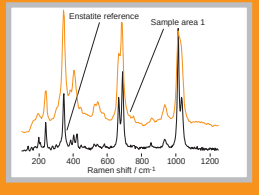


Energy dispersive spectra indicate presence of magnesium silicates.



Raman spectroscopy identifies the silicates present as enstatite ($Mg_2Si_2O_6$) and forsterite (Mg_2SiO_4).

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ampoule cracker (available through EM supply houses) and pour the osmium crystals into your bottle containing the water. Debby Sherman <dsherman@purdue.edu> 23 May 2006

MICROTOMY – JB-4 resin

We are doing a rush job for a client who requires 4.0 µm sections from JB-4 blocks. Our ultramicrotomist extraordinaire, Cheryl, is having a dickens of a time getting the sections to remain flat when removing them from the knife. She is cutting on glass and taking sections from the dry edge with a fine forceps. As soon as the sections leave the knife, they curl and won't uncurl when placed on a drop of water on a slide. Not only is this a rush job in support of a grant proposal, but it requires serial sectioning with no missing sections, and we have, like, no real experience with this resin. Cheryl has tried various sized block faces and different thicknesses for the sections, but nothing is helping. Can anyone help? Randy Tindall <tindallr@missouri.edu> 18 May 2006

Ah yes, the joys of JB-4. I have spent many a pleasant hour cursing the curling sections. In my experience, no change in shape, speed, or thickness makes a difference. I learned to be waiting for the section to start cutting and then either grabbing a corner of it with a fine forceps or using the forceps' tines to hold the corner down on to the surface of the knife while it cut. Then I stop the microtome, remove the section, and re-start the cutting motor. This is tedious and time-consuming. I know you are stuck to the whims of your client whose blocks are already embedded but I strongly recommend any fans of JB-4 consider switching to the generic, and therefore less expensive, butylmethyl methacrylate resin mix of Tobias Baskin. You can cut on water filled boats and use acetone to extract the resin so the sensitivity is better and the sectioning is trivial. Tom Phillips <phillipst@missouri.edu> 18 May 2006

Thanks to all for the advice on sectioning JB-4 resin, with its tendency to fold and curl. I am summarizing the replies below: 1) From Glen McDonald: "For serial sections, which thankfully I haven't had to do with JB-4 in many, many years, I got a tackle box, found in any small parts supplier or fishing supply shop - a clear plastic box with 2x6 or 4x6 array of compartments about 2 inches square. Fill with deionized water or 3% ethanol. As the section comes off of the knife edge, either lay an eyelash across the bottom edge to prevent the curling, or grab with a pair of Dumont #55 forceps. Move the eyelash or forceps along with the motion of the section, then lift the section and drop onto the liquid. Place one section in each compartment to fill the tackle box, then mount them by immersing the slide and bringing it up under the section. *Gently* touch one corner of the section to guide into position. If not gently enough, the section will cling to the eyelash and wad up like a used tissue during a bad cold. There were a couple other variations on this technique of lifting slides up underneath the sections. Off to the Bass Pro Shops electron microscopy department for me! 2) Several people described helping the section come off the block by pulling with a forceps on one corner during the cutting stroke or holding it flat with an eyelash or brush, then flicking the section quickly onto a drop of water or water/ethanol mixture. Dexterity required, methinks. 3) Another repeated suggestion was to put the sections onto drops of water with a little ammonium hydroxide in it. 4) As mentioned above, putting sections into water with ethanol, up to 50%, was mentioned several times, sometimes followed by transferring sections to distilled water afterwards. 5) Don't use JB-4. (My favorite.) 6) Tobias Baskin has published his own formulation of BMM resin which apparently sections much better. He uses DTT in the mix and says he is happy to help anyone with this resin. 7) Humidity should be in the 40-50% range and the block should neither be too wet or too dry or "sectioning is nearly impossible". From Ralph Common. Humidity? In Missouri? Who could have guessed? 8) Related to 7, if the block is too soft, it won't cut well. This one did it for us! Four more hours in the oven solved the worst of the problem. Sections coming off flat and staying flat. These sections did fold when placed on 50% ethanol/water, but did not fold when placed on distilled water. Thanks, Teri Johnson!! This was a crash course in JB-4 emergency microtomy. Randy Tindall <tindallr@missouri.edu> 22 May 2006

MICROTOMY – sections moving around

I've had the strangest thing happen this past weekend and since and no one who I've talked to (some very experienced with thin sectioning) has been able to help. I had been sectioning a block of tissue embedded in Poly/Bed 812 at 50 nm without difficulty using a Diatome diamond knife on a Leica Ultracut microtome, getting beautiful silver sections, spreading then by wafting a toothpick dipped in chloroform over them, and picking them up on Formvar coated slot grids. All has been well until this weekend. Everything was set-up as it had been the previous day. Same knife, same boat water, same toothpicks, same chloroform, same block, etc. As was the case previously, I got beautiful silver sections, but when I went to spread them with chloroform on a toothpick, they ran away as I moved the toothpick close to them, lickety-split. I could chase them around the boat with the toothpick. When I tried to pick them up on a grid, I couldn't. They would just slide off, back onto the water surface in the boat. Naturally, this is happening just as I reach the critical spot in the tissue I'm sectioning. Not to be deterred, I cleaned the boat by washing it with clean water, got new beakers to hold the water I use, and tried again. The same thing happened. At that point I called it quits for the day and hoped it was some strange environmental effect that would disappear the following week. No Joy! When I sat down to section today, the same thing happened. I tried cleaning the knife boat with 0.2% ethanol in water, got new glassware, new chloroform, new toothpicks, etc. Still no joy? So, my question is, can anyone give me some guidance as to what might be going on? I'm at my wits end. Oh, one other thing, if I let the chloroform evaporate off the dipped toothpick, the sections don't run away from it. Thanks for any advice, Steve Kempf <kempfsc@auburn.edu> 18 May 2006

It sounds to me like a static electricity problem. Is your air suddenly much drier? i.e., Did they turn on the air conditioner? We found many years ago that we could block this with those old darkroom "dustfree" brushes with polonium strips in them. I don't know if those are still available. Joel Sheffield <jbs@temple.edu> 18 May 2006

I'll second the notion that your problem is static. In my lab here in Houston, I have the same problem when there is low humidity. On days of high humidity, no "run around" sections. Mannie Steglich <msteglic@mdanderson.org> 18 May 2006

We gave up smelling chloroform a while ago and switched

to using a hot wire. The supply houses sell these, very nice units, but with a little care you could probably make one. They deliver a controlled amount of heat to a thin wire loop. You wave this near the sections and just like chloroform, the heat flattens the sections right out. I realize this isn't a fix for your problem right now, but something to consider for the future. Your liver may thank you! Tobias Baskin <baskin@bio.umass.edu> 18 May 2006

I just wanted to thank everyone who replied to my question yesterday. I too thought static charge might be the problem; however, it seemed strange that if that was the case, the sections didn't "run away" when I put the chloroform dipped toothpick next to them after allowing the chloroform to evaporate. At any rate, late yesterday I dismounted the block I had been having the problems with and mounted new one and, guess what, no problems. Everything worked fine. I didn't have time to re-try the block that I had been having the problem with again following that, so I put it in a 60 degree oven overnight and mounted it this morning. And, as the gods of sectioning would have it, everything was back to normal with no "running away" problems. Go figure? So, if it was static, did just changing the block relieve the charge? At any rate, thanks again for all the suggestions. Steve Kempf <kempfsc@auburn.edu> 18 May 2006

MICROTOMY – Biofilms on latex

We're trying to investigate bacterial biofilms on a latex material (like surgical gloves) with TEM. We couldn't cut the latex material with our regular glass knives. Is there any suggestion about this problem? Necat Yilmaz <nyilmaz@mersin.edu.tr> 12 May 2006

You need a cryo-microtome and work around -40. This gets you below the glass transition temperature for most elastomers and the latex will no longer behave like an elastic material, but a hard brittle glass. You may need to fiddle with temperature and pick-up technique. I've sectioned polymer and used both glycerin/water, mineral spirits/xylene and DMSO/water depending on the temperature and my end goal (Light, SEM or TEM). A diamond knife and boat would be my preferred method. I like to pick up with a "perfect loop" and place on carbon coated grid. Frank Karl<frank.karl@degussa.com> 12 May 2006

LM – field of view

What is the area covered by the microscope objective at the sample? Is there a formula available to calculate the area covered by the objective. N.R.Chakravarthi <chakravarthi@ccmb.res.in> 21 Apr 2006

The formula is a very simple one. The first piece of information that you need is the "field number". This is a value inscribed on the eyepieces. It is the diameter of the field that just the eyepieces see, measured in mm; it will range between 18 and 32 mm. The formula is: Field of view = field number/Magnification of objective* In the simplest case, if you have a field number of 22 mm and a 10x objective, the diameter of the field of view will be 2.2 mm or 2,200 micrometers. There is one caveat (see * above) : if there is any sort of intermediate magnification (e.g., on the Zeiss systems, an "optivar" will allow you to dial in a variety of intermediate lenses providing something like 0.5x, 1x, 1.2x and 1.6x or 2x) or if there is a tube lens with magnification other than 1 (look below the binocular/trinocular body on the intermediate piece), you must multiply the magnification of the objective times that number and use the resulting value. So, if you have the case above, but you have a 2x tube lens or intermediate magnification, then the field of view would be 22 mm/20x or 1,100 micrometers. Barbara Foster <bfoster@mme1.com> 21 Apr 2006

LM - quantitative fluorescence

I am looking for advice for doing quantitative fluorescence microscopy. My focus is on the microscope, camera, and software end of it, rather than the sample prep. Last year, a question about quantitative fluorescence on this list resulted in a number of very helpful posts about things to do, like controlling camera settings and using fluorescence reference slides. However, what is not clear is how to combine all these things together into an appropriate procedure. If anybody could help with any of the following, I would greatly appreciate it: 1. I purchased a set of fluorescent reference slides but have been unable to obtain any documentation or instructions from the supplier for how to use them correctly for fluorescent imaging. 2. When comparing a signal between two samples, is it best to subtract the background from the signal or just compare the two signals? 3. To determine the signal, is it best to use irregular area of interests (AOI) and get a mean or summed value or to use multiple line profiles, or something else? 4. If anyone would be willing to share a step-by-step protocol that they have developed to help users do image capture and analysis of fluorescence correctly, I would greatly appreciate it. Thanks. Anita McCauley <mccaulak@wfu.edu> 25 May 2006

My answer will deal with confocal microscopy: I use to insert my negative control and choose the lasers and detection settings so that I get no signal. Then I insert my samples and won't change the settings. To compare fluorescence intensities, I used to draw a profile line and compare the peaks (maximum intensities). I do this because I observe compact homogeneous bodies. But I suppose I would draw a region of interest (ROI) in the case I had to compare the total intensity of cell cytoplasm for example. It all depends on what and how you measure, there is no general rule I think, it must be adapted to be as "ethical" as possible. Stéphane Nizet <nizets2@yahoo.com> 26 May 2006

It's not really possible to get quantitative measurements of epifluorescence. The gel fluorescence standards commonly used are simply to check to see if your camera/detector and electronics are genuinely linear in response (something quite important to know) - they are no use as standards for calibrating fluorescence within the cell (although you can use things like BCECF in solution at known pH's to roughly calibrate intracellular pH). To properly calibrate cellular fluorescence you need standards that are essentially known concentrations and masses of labelled protein etc.. within cells, not something that's easy to get. You will always have problems with laser power variation, differential bleaching, uneven inconsistent labeling, internal quenching etc... so you will probably never be able to say that one cell has exactly twice the mass (total pixel brightness) or concentration (mean pixel brightness) of a given protein compared to another. However it is reasonable to assume that a very brightly labelled cell has definitely more labelled fluorochrome in it than a poorly fluorescent cell or intracellular region (and you could say something like 'suggesting' x times concentration etc.). Also try measuring something simple like concentrations of fluorescent beads to see how (badly) the image analysis results work out (these

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do have the advantage that you can actually count them as well). You will probably have to use regions of interest to include darker as well as bright areas if comparing regions, plus you can use peak values as described by Stephane (but often the bright sample fluorescence area has to be very clearly defined to one region to get this to work). You can also try things like what % of the cell is brighter than a set grey level. Co-localization (i.e. the % of bright pixels in both the red and green channel across the cell) is also useful. Generally each set of samples require their own image analysis protocols depending on what you want to find out, plus you need simple stats to say if the difference is significant to 95% level. When looking at optical density, this can be more easily calibrated - here you are measuring optical white light transmission though a sample. So you can create black (metal disk), white (free space) and have a selection of known density materials like polythene (grey) etc. in the image. You can therefore assign a grey level to a particular density. We have used this to estimate things like bone density, assuming your can get very evenly sliced samples. Keith Morris <keith.morris@ucl.ac.uk> 26 May 2006

TEM - lead citrate

This is a specimen prep question for everyone out there with EM expertise. We are having terrible success with lead citrate contrast staining. Principally, we suffer from precipitates showing up all over the specimen. On the advice of EM science technical support, we are double distilling our own water (they say Milli-Q is too pure and also is de-ionized which we don't want for EM). Then we make it CO_2 free by autoclaving and capping directly upon removal from the autoclave. We do this the morning of reagent preparation so it doesn't sit in the bottle for longer than it takes to cool down before we begin making up the Reynolds. We make the Reynolds lead citrate according to the protocol listed in Chapter 5 of Bozzola and Russell's Electron Microscopy (2nd edition) (mix 1.33 g lead nitrate, 1.76 g sodium citrate, and 30 ml CO_2 free double distilled water...shake vigorously for a few minutes and then again 5-6 times over the next 30 minutes). Ensure solution is milky white and free of particles. Add 8.0 ml commercially prepared, titrated 1.0 N NaOH. Solution turns clear. Adjust pH strictly to 12.0±0.1 unit. Bring volume to 50 ml with CO_2-free double distilled water. Stopper tightly with rubber stopper and Parafilm until use later that day. When we stain the grids with lead citrate, we make sure to wash well before and after with CO_2 free double distilled water in addition to surrounding the staining plate (Hiraoka kit) with NaOH pellets. In addition, we centrifuge the Reynolds at 5000 xg for 8 minutes prior to use and we 0.2 μm filter it into staining plate. Any advice or thoughts are welcome. What are we doing wrong? What can we change about this protocol to ensure precipitate free staining? Danielle Crippen <dcrippen@buckinstitute.org> 02 May 2006

Are you sure it's lead citrate precipitate? There are many other sources of precipitates and "pepper", as I'm sure you're aware. What kind of sample are you preparing? What buffer is being used? Are you osmicating your samples? We fought a pepper problem for over two years, before finally discovering that adding 2-mercaptoethanol

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to our buffer solved the problem. Randy Tindall <tindallr@missouri.edu> 02 May 2006

I haven't worked in biological electron microscopy for over 25 years. However, I vividly remember a colleague having a terrible time with precipitates from lead citrate staining. Turned out that the problem was his eye-sight. He was extremely near-sighted. To watch his work, his face was only several inches from the staining grid and water rinse. The source of the problem was CO_2 from his breath. Another colleague happened to see his proximity to the stain and suggested the source of the problem. The precipitates disappeared once he isolated his exhaust from the process. Gary M. Brown <gary.m.brown@exxonmobil.com> 02 May 2006

We used special analytical grade NaOH, since it seemed to us that the NaOH was picking up carbonate from the air. The analytical grade stuff came in a sealed glass vial, and made quite a difference. Joel Sheffield<jbs@temple.edu> 02 May 2006

This may be sheer luck, but I've never had trouble with precipitate. I keep my lead citrate, (made up with ordinary distilled water, not specially CO_2 free) in a 50 ml volumetric flask which sits in the same place month after month and is never moved. I don't use the stain for 24 hours after it's prepared, but then just carefully take off what's needed from close to the surface using a glass pipette. I wipe the end of the pipette with a tissue before dispensing the stain and then discard the first drop. I put the drops onto Parafilm in a covered glass Petri dish. No need for NaOH pellets. Finally wash the grids for 5 seconds in a gentle stream of water from a wash bottle. I probably shouldn't admit this but I've had a bottle of stain last over 2 years (the surface of the bottle becomes cloudy with precipitate) and still produce perfect results. Diana van Driel <dianavd@eye.usyd.edu.au> 03 May 2006

We keep lead citrate prepared by Reynolds' method for months in a volumetric flask. We use Whatman #1 to filter and make drops on a Parafilm in a Petri dish containing NaOH pellets. After staining for 1-3 min, wash the grids in water, then water with about 0.01% NaOH and again water. Never had any precipitates. Shashi Singh <shashis_99@yahoo.com> 03 May 2006

Doesn't anybody else use or recommend Sato's lead stain as a more stable replacement for Reynolds Pb citrate? We've used it since the 1970s. 1968 Sato, T.: J. Electron Microsc. 17:158, 1968. 1968 Sato and others: Proc. XIth Int. Cong. on Electron Microscopy. Kyoto. 1986, pp. 2181-2182. Mike Reedy <mike.reedy@cellbio.duke.edu> 03 May 2006

I would like to ask whether your double distilling apparatus overall is made entirely of quartz glass or does it have a distillation container bin made from metal (e. g. copper). I only would like to add this since we had - several years ago - a problem when our distillation apparatus was out of function and on repair for some months and we used bidistilled water obtained from our hospital pharmacy. We had a lot of precipitation problems then, which ended not before we changed to the ddH_2O from the repaired quartz-glass still used formerly. When checking the quality of the "pharmacy"-water later on it turned out to contain a high amount of copper-ions (storage bin was made from copper sheets), which in my opinion perhaps might have had a detrimental precipitating action on the lead-staining performance. By the way: we use Lead Citrate according to Venable & Coggeshall (1965), store solutions in "ultraclean" snap cap-glass-vials and also ultraclean plastic snap cap which are used only for that purpose, that means we take care of any traces of cleaning substances by washing /cleaning also with chrome-sulfuric acid or a modern substitute and take care by ourselves, not a washing machine, to get rid of any resting traces of substances by vigorously washing several times with bi-distilled hot water and a final step with ultrapure water. We found also that intermittent air drying of glass vial/bottle creates probably otherwise insoluble incrustations, so we always keep the stuff in wet condition until the final step of cleaning. Another point we found is that "freshly" made lead citrate solution (Venable & Coggeshall) -if used the same day - will be "more aggressive/more reactive", that means, we decrease staining times (say 30 sec when freshly prepared instead of 2-3 min @ room temperature, e.g. after one week storage in the dark). Avoiding or at least some sort of control for the CO_2-reaction is obligatory in our lab (NaOH-pellets in a Petri dish filled with dental wax, the latter always being melted and flamed after a staining cycle, but perhaps use of virgin, clean Parafilm sheets is the better choice knowing that some disturbing precipitation nuclei also could be present in previously uncleaned, and therefore oily injection needles, syringes, plastic tips, rubber

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stoppers (especially if always one and the same is used) as well as the surface areas where you are staining/handling your grids. In general, our experience is the more steps you are introducing in your schedule to reduce an anticipated precipitate or to inhibit the formation of such one the more you likely will initiate precipitation due to unexpected particle impurities. Wolfgang Muss <w.muss@salk.at> 03 May 2006

I have been using "calcined lead citrate" (apparently a modification of Sato's lead citrate) for a couple of years, and it certainly is much more stable than traditional Reynolds lead citrate. Takamasa Hanaichi et al. (1986) A Stable Lead by Modification of Sato's Method. J. Electron Microsc., Vol. 35. No. 3. 304-306. Jan Factor <jfactor@ns.purchase.edu> 03 May 2006

I've had a couple of inquiries about calcined lead citrate, so I thought I'd send this to the list in case anyone else is interested. To be fair, I learned about this method by perusing the EMS Catalog, which has the formulation. To prepare the calcined lead citrate, the unusual step, I simply went up to our chemistry program and asked them to fire up their high-temp oven (which is in a fume hood) for the day. Once you get a successful batch of calcined lead citrate, and I suggest making a good deal more than you need immediately, it can be stored as a powder in a vial for some time (perhaps indefinitely?). This way, you only have to bake it once, and you can make enough for multiple batches of lead citrate stain. I still use the usual precautions when handling lead stain, such as using NaOH pellets, and I spin down the stain in a tabletop centrifuge before each use. Jan Factor <jfactor@ns.purchase.edu> 04 May 2006

TEM - immunolocalization

We have been doing our pre-embedding immunogold localizations incubations for animal tissue in PBS, 0.2% BSA and 10mM Na azide. I am working now on some EM pre-embedding localizations on plant tissue, for which light microscopy localizations have been done using MTSB buffer (50mM PIPES, 5mM $MgSO_4$, 5mM EGTA, pH~7.0). Is there any reason for which I should not be using this same MTSB buffer for the EM work? Tea Meulia <meulia.1@osu.edu> 16 May 2006

In dealing with plant tissue, EGTA is included in buffers because calcium chelation removes calcium cross bridges and probably some pectin from the cell wall and allows antibody access. Although protocols for doing this originally called for having EGTA in the fixation buffer, in our hands, we get better preservation if the EGTA is included after the fixation as a separate incubation. The removal of the calcium by the same token makes the cell wall weaker. You may find quite distorted tissue. It may be possible to minimize this distortion by including an incubation in mM $CaCl_2$ after the 2nd antibody and before dehydration. Note that buffers with Pipes, magnesium, and EGTA are not microtubule stabilizing (if that is what you mean by MTSB). They are the standard buffers for studying microtubule dynamics in vitro. Tobias Baskin <baskin@bio.umass.edu> 16 May 2006

Quite a while ago I did some pre-embedding immunogold labeling, and fixed in phosphate rather than Pipes buffer, using 2 mM rather than 5 mM Mg and EGTA. I think that you should use the fixative that works best for what you're after, I'd suggest using the same fixative/buffer combination as for the LM work, taking on board Tobias' comments about how EGTA softens the walls causing some tissue distortion if you're not careful. When I did this, I then cut frozen sections, rinsed in PBS then labeled the sections on slides with antibodies in PBS, after the usual blocking in BSA or gelatin. I then embedded the sections in Spurr's (messy) before sectioning for TEM. I did this to get greater penetration of label into tissue, while avoiding the original cut surface of the tissue block. Rosemary White <rosemary.white@csiro.au> 16 May 2006

The buffer you indicated may be fine, although a word of warning may be in place: be careful when using divalent cations like Mg^{2+}. We've never actually tested this for gold conjugates but such ions cause aggregates of gold particles even at very low concentrations. The coating proteins should help preventing that but it may still happen. If that buffer, as Tobias Baskin indicates, is used to open cell walls to antibodies, then it may be sufficient if it is applied only to obtain that effect, i.e. fix, treat with the permeabilizing buffer and then wash with PBS a few times before proceeding using the same protocol that was used for your animal tissue. Jan Leunissen <leunissen@aurion.nl> 17 may 2006

SEM - roots for EM

I've been processing some small plant roots for scanning electron microscopy. I use Glutaraldehyde fixation followed by 0.8% potassium ferrocyanide and 1% OsO_4 - otherwise standard fixation. I get great root preservation, but I've found that the root hairs all look very collapsed. I can send a picture if you like, but was wondering if anyone had experience in this area. Gordon Ante Vrdoljak <gvrdolja@nature.berkeley.edu> 25 May 2006

Have you considered cryo-SEM? This would seem to the ideal method - maintaining the sample in its hydrated state and avoiding mechanical damage that critical point drying can cause some delicate samples. See: http://www.quorumtech.com/Applications/Cryo_Apps_Library/Frozen-Hydrated -Root-Hairs.htm This is quite an old image and at one time (now lost) there was a companion image showing the clean surface after controlled etching (sublimation) of the ice. Mike Wombwell <mike.wombwell@quorumtech.com> 26 May 2006

SEM - imaging sample with magnetic substrate

I have a sample with deposits of an alloy of Sn-Co-C on a magnetic stainless steel substrate (430 stainless steel). I'm using a cold field emission microscope and am having trouble getting high resolution images. Could you suggest what setting I should use to get started? Ryan <lewryan@gmail.com> 15 May 2006

I suppose that you have not only a cold FEG SEM, but a so called "semi-in-lens" type of objective lens (OL) on it too. In fact, the cold or Schottky type of FEG doesn't play a role in the problem. It's only an OL question. That type of OL has a strong magnetic field coming out of it, which envelopes the sample at short working distances, allowing to work at WD0 and to get nice high resolution images of non-magnetic materials, with the "through the lens" SE detector. The field coming out of the OL can be very strong; I have measured values such as 3 kG at WD2 and 10keV primary energy. With lower beam energy the field decreases and at 3 keV, I measured a value of 1.2 kG at WD3 mm, and 0.3 kG

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at WD8 mm. Practically, you have a few solutions, but all are half solutions. First, you must avoid working with short WD. Look at the shortest WD possible without too much astigmatism. Ask the SEM manufacturer at which WD the sample is quite out of the field. If your shortest WD is 2 mm or so, it will be something like 6-8 mm. Second, you must find a primary energy not too low, which would give a poor resolution, due to the perturbation of the primary beam by the field lines in the sample, and not too high, to minimize the field coming out of the OL, which increases with increasing primary energy. In most cases, you'll find a good compromise between 5 and 10 keV. Third, you must play very much with the astigmatism corrections, and re-play again if you move your sample a little bit. And depending on the electronics, you may need to touch up the range limits of the astigmatism corrections. In that case, if you have a CL astigmatism correction setting, you can play on it a little bit, putting astigmatism at the Cl, in the opposite direction, to gain some margin of the OL astigmatism settings. It's not a clean way to work, but in may help. Fourth, take the smallest sample possible, not too thick, not too wide, and fix it very securely on the holder. It may fall from it and stick to the OL! Fifth solution: buy another SEM for that kind of samples! If you have a nice picture at x50000, you can be satisfied! More depends on your particular situation, sample and SEM. Jacques Faerber <jacques.faerber@ipcms.u-strasbg.fr> 16 May 2006

I would suggest setting the specimen at a working distance of at least 15mm so as to be outside the field of the lens. What may also help is to lower the kV as this will also lower the lens field. Of course the kV level will depend upon what you are asking of the specimen? If you wish to examine the TRUE surface <5kV will be ideal. If you wish to investigate the sub surface detail 15kV backscatter would probably be a good starting point. Do not try to use an upper detector if fitted to the instrument as this will require your being close to the lens. Lower detectors in a twin detector system require a higher probe current (weaker C1) than that used for an upper detector. Good luck. Steve Chapman <protrain@emcourses.com> 16 May 2006

In addition to all the other suggestions about small sample size, out of the lens field, etc., also try running your sample through a degausser just before putting it in the SEM. Any residual magnetism is going to adversely affect a high magnification image. I can remember (too many years ago) swearing at the SEM I operated (because my resolution was terrible, then remembering that my sample was a piece of carbon steel. After degaussing, the resolution was fine. Ken Converse <kenconverse@qualityimages.biz> 17 May 2006

SEM - ultrafiltration membranes

We are trying to take SEM images of 30 to 500 kDa ultrafiltration membranes. The ultrafiltration layer is regenerated cellulose. The problem we have is that drying the membrane leads to collapse of the pores. Is there a method, e.g., use of super critical carbon dioxide, that can be used to prevent collapse of the pores when the membranes are dried? Ranil Wickramasinghe <wickram@engr.colostate.edu> 21 Apr 2006 1

How big a territory do you need to image and how big a difference in topography is there on your membrane? This may be another place that AFM can help. We can run these samples in a liquid cell to maintain the moisture content, if the differences in topography are not too great. Based on some general polymer studies we've done, I would think that a phase image would be very revealing. If you would like us to run a test sample, please contact me off line. Barbara Foster <bfoster@mme1.com> 21 Apr 2006

We have critical point dried ultra filtration membranes (but maybe not cellulose), however the pores in the top skin are still very difficult to resolve even by TEM because of insufficient contrast. By SEM, we have been able to obtain images only of the support structure but not of any pores in the outer skin. This is much more of a TEM than SEM application. Some years ago we had a system in which we precipitated silver chloride into the pores, did a low acid GMA embedding (to avoid an alcohol dehydration step), cryo thin sectioning, and thought we had decorated the pores. But that to me is the only way one could really resolve the pores in the top skin. I have no idea if this approach could work for your system, you would have to just try it and find out. Charles A. Garber <cgarber@2spi.com> 23 Apr 2006

SEM – red blood cells

I need to look at some RBC's using SEM. Does anyone have a favorite protocol for preparing them? Tom Phillips <phillipst@missouri.edu> 16 May 2006

We've used poly-l-lysine cover slips and standard fixation/dehydration procedures with good success. I believe some people have used HMDS successfully, too. Randy Tindall <tindallr@missouri.edu> 16 May 2006

Human red blood cells respond nicely to fairly routine fixatives. We use cacodylate with Ca^{2+}, Mg^{2+}, and NaCl added as buffer system but biological buffers such as PIPES should work also. However, be aware the RBC's from other animals have different osmotic requirements and may crenellate easily when the human RBC's are fine. We ran into real problems with mouse RBC's a while back. Only sure way to avoid problems is to check and balance osmolarity of fixatives for the specific host RBC's. Debby Sherman <dsherman@purdue.edu> 16 May 2006

I would only fix them in 1.0% buffered glutaraldehyde at room temperature for 2 hrs. Then wash the cells in distilled water 3x and resuspend in ddH_2O. Take a drop of the solution and place on discs of aluminum foil 5mm in diameter attached to studs. Let air dry at room temperature for 1-2hrs. Coat the specimens. That way you won't need to use HMDS. Karen Bentley <karen_bentley@urmc.rochester.edu> 16 May 2006

EM - platinum nanoparticles

Today's challenge is trying to image platinum nanoparticles in an agarose matrix, in order to see how they distribute themselves and to get a size distribution. TEM and/or SEM are possibilities. So far, my check of the literature finds tons of stuff on nanoparticles in electrophoresis gels, but none of it is relevant, since the particles are removed from the gels and put back into a liquid medium. The one article I found that is comparable to our problem used thin-sectioning and we have tried that. We have also tried melting the agarose, dipping grids into the particle/agarose mix, and then rinsing the grid in hot water to thin the gelatin out. We can get images in the TEM, but the results are inconsistent when repeating with

the same sample. Also, there is the chance that the hot water and melting are re-arranging the particles. We have tried thin sectioning the dehydrated agarose with particles, but finding the particles in a given thin section is a crap shoot with long odds. Also, we may be cutting through aggregations we want to see. We have tried viewing carbon-coated dehydrated agarose with particles using backscattered electrons in our FESEM. This gives images with particles, but only those on or right at the surface are imaged clearly enough for good size data. Plus, we can't see far into the agarose for good distribution data. We are going to try increasing the concentration of the particles to increase chances of getting them reliably in thin sections, and we will also try putting the melted mixture on cover slips in a thin layer and re-trying the BSE imaging after carbon coating. The latter still has the potential problem of redistributing the particles, however. We could also try doing large thick or semi-thin sections and viewing them in BSE imaging. However, if someone out there has viewing nanoparticles in agarose down to a fine art, we, as usual, would be delighted to hear about it. In the meantime, I will continue to search the databases. Randy Tindall <tindallr@missouri.edu> 02 May 2006

Thick or semi-thick sections in TEM would be my choice probably since I have a 300 kV TEM. If you can get to a high-pressure freezer, I would suggest using that to prepare your specimens, followed by freeze-substitution and resin embedding. Assuming that you do not need to image the strands of agarose, just section the embedded specimen and observe. Bill Tivol <tivol@caltech.edu> 02 May 2006

LM – fluorescence in plastic resins

I would like to localize a fluorescent molecule in the transverse plane of a cell monolayer (not from above). We have no material for the preparation of histological sections, but we have all necessary for TEM. I wonder if I could not embed the monolayer in Epon, then cut transverse semi-thin sections and observe in epifluroscence. Would Epon hinder fluorescence? Could it be done in another resin than Epon? Stéphane Nizet <nizets2@yahoo.com> 07 Jun 2006

Not so weird, but it might not work. The two things to worry about are autofluorescence and loss of your fluorochrome. If you have some of your cells (unlabeled) in Epon you can check some sections under a fluorescence microscope and see about the autofluorescence. I think the loss of fluorescence is a more serious problem, either because it is extracted during dehydration/infiltration or quenched by the embedment. You may have to embed a labeled sample and find out the hard way! Tobias Baskin <baskin@bio.umass.edu> 07 Jun 2006

Regarding the idea of localizing fluorescence label in Epon, I would suggest that you not use Epon. There are autofluorescence problems (at least in our hands, using Spurrs resin). However, I think you may be happy using LR White, polymerized at 60C. We've done some similar work here and have been very impressed with the results. Doug Keene <drk@SHCC.org> 07 Jun 2006

Success will depend upon the fluorophore. FITC and TRITC

will lose their fluorescence. Alexa fluor dyes and cyanine dyes will work well. Actually, I think with reduced photobleaching. Autofluorescence will depend upon imaging modality. Spurr's looks great under the confocal, but the autofluorescence creates an impossible haze with epi-fluorescence on thick samples. Deconvolution will clean up the haze. 3 micron sections looked very good. The new formulation of Spurr's is too brittle so I'm going to try Eponate 812 or something similar for my next round of embedded fluorescence. I tried Histo-Resin and found the autofluorescence was noticeable under confocal, but I didn't try LR-White. Reference: Hardie, MacDonald, Rubel, Brain Res. 1000:200-210, 2003. Glen MacDonald <glenmac@u.washington.edu> 07 Jun 2006

Thank you for your numerous answers. Lots of them show a concern about autofluorescence, but I must say that I was more concerned about the quenching by Epon and the processing steps: dehydration and embedding. I wondered if and how it could "damage" the fluorochrome. We use Epon 812 (glycidether 100) and we just cut empty blocks at 300 to 500 nm thickness to observe the autofluorescence with the "green" filter (our fluorochrome is Alexa488). Actually there *IS* some autofluorescence, but not dramatic even with a thickness of 500nm. I don't think it will perturb the observation, but it depends of course how well the Alexa will sustain the processing. An excellent remark was made, pointing out that glutaraldehyde must be avoided because it brings autofluorescence. I will definitely try light fixation (4% PFA for 20 min), fast dehydration in alcohol and direct embedding in Epon. Another concern is the localization of the cells and cell compartments. Do some of you have an idea how use DIC or phase contrast with cells embedded in Epon? Could I just use general staining protocols hematoxylin-eosin before embedding in Epon? Stéphane Nizet <nizets2@yahoo.com> 08 Jun 2006

Don't use eosin...it fluoresces very intensely (in the usual "rhodamine" wavelengths). In fact, the slide I use when teaching people how to use the confocal is a standard paraffin section stained with H&E. It never seems to bleach, and it fluoresces like mad at 488 (the connective tissue), 543, and 633. I've looked at semi-thin resin sections with DIC...it works. Leona Cohen-Gould <lcgould@med.cornell.edu> 08 Jun 2006

I wonder if the autofluorescence might be due primarily to the MNA (aka NMA) in the usual Luft formulation? When I found that NMA caused the cured resin to react horribly with MnO_4 section stain — (useful because the MnO_4-Pb staining sequence gives much more contrast than any other stain we've ever tried) — I learned to avoid MNA and just empirically readjusted DDSA and Epon ratios empirically for a firm enough cured resin, needing no MNA, yet compatible with MnO_4 staining. However, I soon abandoned Epon and Spurrs of all kinds in favor of an Araldite 506-DDSA-DER 736 mixture (10:15:2 gm by weight) because it sections thinner and accepts Mn-Pb staining with less graininess. So that Araldite mix is what I will try soon with Alexa 488-phalloidin. Mike Reedy <mike.reedy@cellbio.duke.edu> 08 Jun 2006

Interesting thought about the NMA, and your Araldite recipe looks worth a try. I had dropped the DMAE in Spurr's to 1/2 and then to 1/4 of the original recipe, thinking that it might generate radicals reacting with the fluorophores. The result was slightly reduced autofluorescence after a 3 day cure time at 60°C. No apparent effect on the fluorophores themselves, but didn't spend any time measuring. FYI, alcohols will cause phalloidin to dissociate from the actin. Maybe it will survive a higher alcohol, propyl or butyl, but an actin antibody would be a better choice if needing to dehydrate the sample. Glen MacDonald <glenmac@u.washington.edu> 08 Jun 2006

TEM - viral particles

I need to visualize viral particles grown in cell culture, but I do not have any experience with that (only with tissue samples). Could you give me any advice (collection of cells, fixation, embedding)? Wim Van den Broeck <wim.vandenbroeck@UGent.be> 08 Jun 2006

While on the Medical School Faculty of USC, Los Angeles I did extensive studies of hepatitis B virus in liver explants. The technique is straightforward. Remove cells using a soft spatula or a pipette depending on whether or not the cells are attached to the surface of the culture container. Place the cells in a centrifuge tube containing fixative. Carry out the dehydration and embedding fluid impregnation process in the centrifuge tube spinning between each stage. Finally transfer the embedding mixture with the cells into a BEEM-type embedding capsule and spin the cells down into the tip. Ted Dunn <drteddunne@yahoo.com> 08 Jun 2006

It depends if you need to visualize the viral particles inside the cells or not. If so, I would consider the usual protocol for classical cell morphology. I already observed viral particles in cells I observed for other purposes. If you need the viral particles alone, you have 2 solutions depending on the concentration of the virus in medium. 1) If it is concentrated, you just collect the supernatant, centrifuge 5 min at 5000 RPM to pellet the cells and cell debris, and collect the supernatant again. 2) If it is diluted, you have to find an ultrafast centrifuge and perform an additional centrifugation to pellet your virus particles and concentrate them. Then you can just do a negative staining (PTA worked well with rhinoviruses for me). You will find tons of protocols on the net. Stephane Nizet <nizets2@yahoo.com> 08 Jun 2006

MICROSCOPY – imaging protein crystals

Any suggestions on the best way to image protein crystals? They are 20-100 um in length and very stable in high molarity salt solutions or PEG solutions, but dissolve readily in less dilute aqueous solutions. We would like to avoid chemical fixation. Joe Neilly <joe.p.neilly@abbott.com> 12 Jun 2006

CryoTEM is a good method if the crystals are thin enough--10s of nm. They would appear lighter against the darker buffer if the salt concentration is high enough, but they might have very little contrast in a PEG buffer. If the crystals are well-ordered, you should see strong diffraction spots and strong spots in the FFT of an image. Plunge freezing would be good for thin crystals. Bill Tivol <tivol@caltech.edu> 12 Jun 2006

Why not try an ESEM in the near liquid state? They sound more than large enough. Nestor Zaluzec <zaluzec@aaem.amc.anl.gov> 12 Jun 2006

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