NETNOTES

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Selected postings from the Microscopy Listserver (http://microscopy.com) from 10/11/06 to 12/15/06. Postings may have been edited to conserve space or for clarity.

SAMPLE PREPARATION – buffers for fixation

Perhaps we could exchange our views about the optimal buffers to use during fixations for TEM. Cacodylate is a traditional buffer, but it is not really healthy and perhaps it is time to update our knowledge. I have read that PIPES offered the lowest extraction but on the downside a less efficient buffering. Phosphate buffer does not allow the use of Ca^{2+} and Mg^{2+}, which could help stabilize some structures. HEPES buffer...well I don't know the downside, perhaps it offers only advantages but it is used pretty scarcely by the electron microscopists. Some other parameters can be taken into account: the storage, bench life, morphology, immunology, plants, animal cells and so on. Let's talk about salts! Personally I use cacodylate for classical fixation of cells for morphology because it has also been used in the laboratory but I am eager to change. There is certainly some room for improvements in the morphology (especially of the membranes). HEPES seems to be a good candidate. Stephane Nizets <nizets2@yahoo.com> 11 Oct 2006

Buffers have been on my mind lately too. In this laboratory, we have switched our buffer system over to Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium. In large part this is a matter of convenience. We do quite a few immunocytochemistry experiments, labeling culture cell membranes with a variety of antibodies, and we dilute these antibodies and subsequent colloidal gold conjugates with DMEM. The cells remain viable during incubations in antibody and secondary conjugates. It seemed reasonable for us to continue with DMEM during fixation, and we therefore dilute our cacodylate buffered 3% glutaraldehyde + 3% paraformaldehyde stock solution with DMEM and also our OsO_4 in DMEM. We have been pleased with the quality of the fixation and now we also use DMEM for buffering fixatives of tissues with equally pleasing results. But I have to admit to a nagging feeling that I am breaking some kind of microscopy law and would appreciate comments. Douglas R. Keene <drk@shcc.org> 11 Oct 2006

For 40 years we have used the physiological or experimental buffer solution, often with MOPS as the pH buffer, as our main vehicle for primary fixatives composed with aldehydes, aldehyde-tannic-acid, or tannic acid alone, because we believe this offers the best hope for preserving native structure of various muscle structural states (but also effective with other cells and tissues). "Physiological" mostly implies proper ions: for extracellular salines (Na^+ at ~100-145 mM; K^+ absent or below 2 mM; Mg^{++} and Ca^{++} at 1-4 mM); or intracellular rest-state salines (EGTA and no inadvertent Ca^{++}; Mg^{++} at ~5-7 mM; K^+ (or Na+) up to 150 mM if desired; chloride, propionate, acetate, or methane-sulfonate as anion). So for intact frog leg muscle (or insect flight muscle!) we used frog Ringer solution. For mammalian muscle we used mammalian Ringer, with either phosphate or MOPS buffer, adjusting pH with a "dirty" (fixative only) pH electrode to discover the amount of alkali or acid needed to restore original pH after adding various fixative at various concentrations. The presence of up to 3 mM calcium and up to 10 mM magnesium, as dictated by various buffers, was essential in Ringer recipes, and presented no problem. We were happy because the fiber x-ray diffraction pattern and the EM itself showed these fixatives to give good preservation of various distinct pre-fixation native structural states, and I think this was not so for our brief long-ago trials of phosphate or s-collidine buffer. We usually ignored adjustments to osmolarity, but if tried, was always done by adding the osmolyte to the formulation, sucrose top Ringer, 500,000 MW dextran to intracellular buffer. For secondary fixation, buffer choice need not be physiological. For aldehyde-only or aldehyde-tannic acid (TA) primary fixation, post-fixing in OsO_4 has routinely employed a Maupin-Pollard good-for-actin buffer (0.1 M phosphate, pH 6, with 10 mM $MgCl_2$, used ice cold). Most of our work has been with chemically demembranated ("skinned"; by detergent-glycerol) muscle of rabbit, frog or insect, so we chose various composition of intracellular buffer to keep the organelles happy and in the desired functional state of the muscle machinery (rigor, Mg-ATP relaxed, Mg-ATP-Ca activating, or modified with nucleotide analogs (like AMPPNP) phosphate analogs (like vanadate). We have for 30 years used nothing but 20 mM MOPS buffer (relatively inexpensive, and good for pH 6.5-7.5; we prefer 6.8 for insect) for both the physiological and fixative versions of our solutions, typically with 5 mM each of additives like $MgCl_2$, EGTA, Ca-EGTA, ATP, Na azide (stops microbial growth, arrest ATPase consumption by mitochondria). For years we strictly used potassium salts in preference to sodium to honor the cell's intracellular habit, but lately have slipped away from this dogma without bad results, drifting to more frequent use of sodium (as hydroxide to adjust pH of EGTA, ATP, MOPS, etc. Sometimes we use salt to approximate intracellular ionic strength, typically using KCl (now NaCl) at 100 or 150 ml, but we often use no extra salt at all, because its presence or absence has so far made little or no difference to the fiber x-ray diffraction patterns which are our gold standard for native and preserved structure. We have always steered clear of Tris because glutaraldehyde reacts with it, changing pH, and because Tris-buffered pH alters with changing temperature. 0.2% tannic acid alone in the physiological (intracellular type) buffer works well on skinned cells to fix all but soluble components. It is blocked or complexed so it becomes unavailable for fixation when Triton X-100 or PVP are included in the same solution. TA-fix should be followed after rinse-out by uranyl acetate (typically in deionized water) or OsO_4 (in Maupin-Pollard good-for-actin buffer) as a secondary fixative. Mike Reedy <mike.reedy@cellbio.duke.edu> 11 Oct 2006

Mike Reedy mentioned that he uses MOPS for a buffer at pH 7 for fixations. I have always used PIPES. Has anyone ever compared the performance of these two Good buffers around neutral pH? Are there reasons to choose one vs. the other? Tobias Baskin <baskin@bio.umass.edu> 11 Oct 2006

The ability of a buffer to maintain pH depends on how many groups there are on the molecule that can take up or release H+ and the pKa of each of these groups. For most buffers there are one or a few of these groups that have different pKa's; e.g. PO_4^- with pKa's of ~12, ~7, and ~3. I don't know offhand what pKa's are available for either MOPS or PIPES, but the closer a pKa is to the pH you want, the better the buffer will be at maintaining that pH. The reason for this is easily seen from the mass action law, which can be written K = [A][H]/[AH] from which it can be seen that for [H] = K, [A] = [AH], and if [H] = K, then pH = pKa. When the protonated and unprotonated species are at equal concentration, the addition of a small amount of acid or base will change the [A]/[AH] ratio less than if they are at unequal concentration. Therefore, the performance of either Good buffer will equal that of the other one if the desired pH is the same amount away from either pKa. Another reason to choose one over another is if one of them affects the process you are trying to perform. It may be that one will react with your fixative which I do not think is the case for MOPS or PIPES and either glut or OsO_4. Bill Tivol <tivol@caltech.edu> 11 Oct 2006

This discussion takes me back to my doctoral research, in which I was concerned about the morphology of dissociated chick retina cells as they re-associated to form tissue-like aggregates. (Develop. Biol. 23:36-61, 1970). As part of that study, we fixed suspensions of cells in various concentrations of phosphate buffer, and measured the cell size distribution in the population with a Coulter Counter. We then chose to fix in 0.08 molar phosphate with 2.5% glutaraldehyde since that distribution matched the result with living cells. It is clear that one can't just calculate

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osmotic strength for these systems, since the fixative alters the membrane behavior in a non-linear way during the fixation process. Joel Sheffield <jbs@temple.edu> 11 Oct 2006

I have used serum free media for many cell culture fixations. I think it works fine. After fix I go into cacodylate so that I do not have any interactions with my tissue culture medium and any post-fix steps. David Elliot <elliott@arizona.edu> 11 Oct 2006

An important topic. I'd like to step in with a reply to just the point about "Could aqueous solutions of osmium tetroxide be used in post-fixation steps?" Yes. Not only have I known folks who make their OsO_4 in distilled water for TEM, I have studied cultured cells in high-resolution SEM using OsO_4 in distilled water, not buffer, for post-fixation. Usually with 1% monomeric tannic acid (for plasma membrane preservation), but not always. Works fine. The colligative properties of the plasma membrane seem to be pretty much eliminated by fixation. pH isn't the only confusing issue here, osmotic properties are, too. 3% glutaraldehyde is about 300 mOsM itself, more if there are impurities and after adding buffer. Yet, this hypertonicity doesn't seem to cause osmotic problems. Worse, something I've never seen discussed: what are the osmotic effects of different pH's? Changing the pH will change the flow of ions and water in and out of cells. If a fixative is present as the pH changes, then the changes in the membrane proteins affecting the osmotic properties will be fixed. So potentionally, a correct pH may in fact not be the physiological pH of the living cell, but the pH that maintains the correct osmotic pressure(s) during fixation. Phil Oshel <oshel1pe@cmich.edu> 12 Oct 2006

SAMPLE PREPARATION – high pH buffer for fixation

I have learnt a lot about buffers used in fixative and in general about buffers in sample preparation. This is very helpful discussion. We work with mollusks (oysters) and our oysters are reared at 860 mOsm of sea water, and a pH of near about 8.2. The cells I look at in SEM/TEM have very fine pseudopodia and the preservation of this structure is my primary concern. Could any one please suggest a buffer that works best at a pH higher than 8? After making up the buffers we measure the osmolality and adjust it, but now from the discussion I feel the need to know a buffer that can work at a higher pH. Neeraj Gohad <ngohad@clemson.edu> 12 Oct 2006

In the late 1970's I worked with Mitilus and Thyone sperm in the lab of Lew Tilney. At that time the primary fixative that I used was 1% glutaraldehyde, if I remember correctly, in filtered sea water. The samples were then washed in Millonig's phosphate buffer and post fixed in 1% OsO_4 in phosphate buffer at pH 6.2 on ice and in the dark. It worked well in this system. Pat Connelly <connellyps@nhlbi.nih.gov> 12 Oct 2006

I don't have access to a table of the pKa's of all the Good buffers, but I seem to remember that there are some with pKa's in the range you want. Failing that, the Handbook of Chemistry and Physics gives the pH for a 0.1N solution of $NaCO_3$ as 8.4. Of course, many cations will precipitate bicarbonate. Morpholine has a pKa of 8.33, several dipeptides have pKa's in this range with leucylglycine at 8.28, and many alkaloids have pKa's near 8.2, but explaining why you want morphine (8.21), strychnine (8.26), or codeine (8.21) may not be worth it. Bill Tivol <tivol@caltech.edu> 12 Oct 2006

Terrific little article on buffers, Good (= Good's) buffers with table or working range and pKa's at wikipedi at http://stanxterm.aecom.yu.edu/wiki/index.php?page=About_buffers. Mike Reedy <mike.reedy@cellbio.duke.edu> 12 Oct 2006

There's also a useful booklet from Calbiochem called (you guessed it) Buffers: A guide for the preparation and use of buffers in biological systems. I have the "new" 1995 edition, but it has all the Good buffers, recipes, comments about other issues such as cation chelation, notes on protein pI, etc. Rosemary White <rosemary.white@csiro.au> 12 Oct 2006

SAMPLE PREPARATION – fixation of lymphocytes

I have been trying to help a student do cryo-SEM on cultured T-cells. He extracts human blood, collects T-cells, cultures them in a medium containing serum and then exposes them for varying lengths of time to chemokines. After culturing the cells were washed briefly in PBS to remove serum then fixed in 1% glutaraldehyde in PBS for 15 min then applied to a filter where as much liquid as possible is removed immediately before freezing in nitrogen slush and carrying out cryo-SEM. We have the problem that the cells appear in the SEM round, smooth & without pseudopodia! We have previously done TEM on the same cells (different extraction) and had good results showing cells with interesting pseudopodia. The difference with the TEM prep was that the fixative was added to the culture medium and the cells fixed for 2 hrs at room temp then overnight in the fridge. We intend to try conventional SEM on the cells next using the initial TEM prep method then critical point drying after dehydration. I am concerned that serum will be fixed to the surface of the cells if they are not washed prior to fixation. I would appreciate any advice on the above. Ursula Potter <u.j.potter@bath.ac.uk> 12 Oct 2006

Try skipping the glutaraldehyde fix. You're cryo-fixing the cells, so a chemical fixation should not also be needed. I assume the cells are cultured on something nice and thin and heat conductive, yes? Little metal coupons, Formvar-coated TEM grids, bits of coverslip sized to the SEM stage before culturing, or the like. Rinse the cells briefly with serum-free buffer, best is the same buffer they're cultured in, just without serum. Make sure the temperature of the wash buffer is the same as the temperature of the incubation medium. Remove from the buffer and immediately plunge into the slush nitrogen. Don't blot off the excess buffer too much, just a quick touch of the edge of the grid, etc. There won't be much, and rapid plunging into slush nitrogen freezes fast enough that a thin layer of buffer won't interfere with good freezing. The buffer water gets vacuum sublimated during cryo-coating, and if you're looking at the cells uncoated, they can still be sublimated. The conventional fixation/critical point drying method also works well, but I strongly suggest adding 1% monomeric tannic acid to the 1% glutaraldehyde as well as to the osmium (although OsO_4 is not necessarily needed for high-resolution low-voltage SEM of cells). You are correct, the serum must be washed away however you fix the cells. Otherwise, you get these wonderful, stringy strands of serum proteins obscuring everything. Especially with chemical fixation. Phil Oshel <oshel1pe@cmich.edu> 12 Oct 2006

SAMPLE PREPARATION – cells grown on collagen gels

I have been having serious problems embedding cells grown on collagen gels. I have tried increasing my ethanol dehydration and infiltration steps. I have tried different resins (Spurr's and LX112). I still end up with gumdrops. These are not thin coatings of collagen as you would use to get finicky cells to grow on coverslips or dishes. These are actually cushions of collagen cast within the confines of cloning rings and probably about 30-50 micrometers thick when fully hydrated. Why should these be so much more problematic than tissue? The PI has also given me the same cells grown on fibronectin, and those are fine. Unfortunately, the cells behave a little differently on FN, and the structures the lab is studying aren't as abundant. Leona Cohen-Gould <gould@med.cornell.edu> 25 Oct 2006

I have had some success with collagen gel in the TEM. Tips: Small size, and dehydrate. Days and days... lots of changes... collagen likes water, and that's where we've had the problem both in the critical point drying and with the Spurr's. We found it was impossible to dehydrate the collagen while it was attached to a coverslip, it held onto the water very strongly. I don't know if this helps, but other than doing many 100% ethanol changes prior to infiltration with the resin, the samples weren't treated any differently and we had decent results. Geoff Williams <geoffrey_williams@brown.edu> 5 Oct 2006

If water is the problem, you might try one of the plastics that do not mind some water. David Elliott <elliott@arizona.edu> 26 Oct 2006

Thank you all for your hints, tips and insight. The general consensus is that collagen gels are just mighty sponges that require very rigorous and prolonged dehydrations. Anything that helps accomplish that is good: releasing the gels from the dish, cutting them into smaller pieces, *en bloc* stain-

ing with uranyl acetate, using multiple changes of propylene oxide before the resin, using a resin with more "tolerance" for a little water. The PI was concerned that the integrity of the cell monolayer would be compromised by removing the gels from the dish and/or cutting them into pieces. I think that I've convinced him that slight damage to a monolayer that I can then cut and we can look at is vastly preferable to what we have at the moment. Leona Cohen-Gould <lcgould@med.cornell.edu> 26 Oct 2006

SAMPLE PREPARATION - preservation of microbes in external mucous

Sometimes I want to get rid of mucous, but sometimes I want to preserve it. In this case the researchers want to look at microbes in the mucous surrounding corals, as well as microbes within the coral tissue with TEM. I have had pretty good success decalcifying corals for TEM, but it's been a long time since I wanted to keep slime. I know people use ruthenium red and Alcian blue to stain mucous, but do these preserve it throughout processing for TEM as well? I also have some ruthenium tetroxide in the fridge from the early 1990s; I think it was once used for preserving slimy biofilms. Tina Carvalho <tina@pbrc.hawaii.edu> 28 Nov 2006

Besides the things you mentioned, there are techniques for "non-aqueous" fixation to preserve biofilms using fluorocarbon solvents, such as Fluorinert FC-72, which is manufactured by the 3M Company. Call them for distributor information. Also high-pressure freezing and freeze-substitution are options, and dehydration in HMDS can help retain mucins for SEM studies. References to check: Allan-Wojtas et al. (1997) Micros. Res. Tech., 36:390-399; Sims et al. (1991) Biotech Histochem. 66(4):173-80; Sanchez et al. (1997) J Comp Path. 117(2):165-70; Sims and Horne (1997) Am J Physiol 273: L1036-L1041; Bock et al. (1999) Biotech Histochem 74(5):244-7. Rand Tindall <tindallr@missouri.edu> 28 Nov 2006

SAMPLE PREPARATION – charcoal and wood

I have a few charcoal and virgin wood specimens with sizes of 100 x 100 x 50 mm and 30 x 30 x 100 mm. How should I prepare these specimens before bringing them to the lab for SEM scanning? Can the SEM chamber accommodate such specimens? Should I reduce the size? Winnie Wino <winne.wino@gmail.com> 11 Oct 2006

The biggest problem with wood and, to a lesser extent charcoal, is that it is very porous, has water in its structure and will out-gas a lot of water vapor. Fresh concrete has the same problem. I have had to cut my wood samples down to less than a 10 mm cube in order to get the SEM to pump down to high vacuum. If you are looking at the wood samples in a variable-pressure SEM it should not be such a big problem. Most modern SEMs, unless they are very high resolution, will look at a 100 mm square sample; it is the pumping capability that might be a problem. Mary Mager <mager@interchange.ubc.ca> 11 Oct 2006

SAMPLE PREPARATION - embedding wood

How would one go about embedding wood? I am having trouble orienting the end grain in a BEEM capsule. Owen Mills <opmills@mtu.edu> 07 Dec 2006

Seal the BEEM cap with some nail polish (or whatever) and turn upside down. This will let the long axis of the wood be parallel to the surface. If you want endgrain end on, after polymerization, use a saw of some kind and cut a few mm "slice" off the end and then trim and mount upright on a blank block. Superglue bonds to epoxies really tightly so this works, though admittedly inelegant. You can also buy BEEM style caps (they are in fact TAAB capsules) that have flat bottoms so you don't have to seal the tops and flip. Tobias Baskin <baskin@bio.umass.edu> 07 Dec 2006

SAMPLE PREPARATION - propylene oxide vs. acetone

What are the opinions on using propylene oxide vs. acetone mixed with resin in the final steps of resin infiltration of TEM processing? We use propylene oxide and have for years. The question came up as I am processing a piece of rubber tubing and am concerned the propylene oxide may eat away at it? Rob Clark <robert.clark@sharp.com> 16 Oct 2006

We have switched almost entirely to acetone for our dehydrating and infiltration steps. It works just fine with our various permutations of Epon (EMbed), Spurr's, and Araldite and allows us to completely avoid the carcinogenic and hard-to-handle propylene oxide steps. We also use it occasionally as a transition between ethanol and resin in the rare cases when we want most of the dehydration done in the somewhat less-extractive ethanol. We use microwave processing for the vast majority of our samples, but it also works with longer procedures. We have no experience with embedding rubber tubing, however. Randy Tindall <tindallr@missouri.edu> 17 Oct 2006

Actually you may simply skip this step. You can do the infiltration steps with a mixture of resin and 100% ethanol. Stephane Nizets <nizets2@yahoo.com> 18 Oct 2006

I would be careful about this - I have found that traces of alcohol can adversely affect polymerization. I do have to use ethanol/resin mix when processing cells that have been cultured on plastic dishes, but always give extra changes in pure resin in a vacuum incubator to ensure removal of trace ethanol. It is our normal practice to use acetone as a link reagent, having changed from using propylene oxide some years ago. Alastair McKinnon <a.d.mckinnon@abdn.ac.uk> 18 Oct 2006

SAMPLE PREPARATION - LR White flat embedding

I have some fibroblasts which were grown on glass coverslips and fixed, dehydrated and infiltrated with LR White. I put a piece of Aclar film over the top and polymerized the resin, but now I am having a very difficult time getting the LR White to separate from the coverslip so that I can glue it to an empty BEEM capsule. Does anyone know any good tricks? Dennis McDaniel <dmcdaniel@usuhs.mil> 31 Oct 2006

Two thoughts; 1) not helpful now, but have the cells grown on Aclar rather than glass in the future 2) try dry-ice on the glass. This often helps remove the glass, sometimes it just shatters the glass leaving the plastic behind. I have had mixed results. David Elliott <elliott@arizona.edu>31 Oct 2006

When separating glass from resin I use liquid nitrogen. I dip the coverslip about halfway and hold there for just a few seconds. Then I hold the slip and resin combination between my finger and thumb to begin warming. It seems the warming happens at a different rate and causes the layers to separate. You can then slip the glass away from the resin. Sometimes the glass shatters, but you can still slip it off in pieces. In future, try growing your fibroblasts on Thermanox coverslips. It works very well and the cells grow just fine. Invert the coverslip over a drop of resin on Aclar and polymerize. The coverslip and Aclar are removed easily from the resin. Even if the resin sticks slightly to the coverslip the layer is so thin that you can simply use a razor blade to cut and remove the precise area of the cells that you would like to look at. The rest of the cells can be saved for another time. Jo Dee Fish <jfish@gladstone.ucsf.edu> 31 Oct 2006

SAMPLE PREPARATION - LR White contrast

I have been using LR White resin for immunolabeling. I have problems with contrast in my samples. Sometimes several sections will be too poor a contrast to get pictures and sometimes part of a section will be just adequate contrast. I understand too long an infiltration time can give you contrast problems. I've cut this down but can only cut it back so far as my material is difficult to get embedded. I am using a TEM for my immunolabeling work. M. Kelly <mkelly@fs.fed.us> 09 Nov 2006

One way to add contrast without affecting antigencity too much is to add ~0.2-2% tannic acid to fix or freeze-sub solvent, followed by a similar concentration of ferric chloride (rather than OsO_4). How strong a concentration you use and for how long varies quite a bit with tissue, and I've only ever processed plant tissues - which often need reasonably long (2 days to a few weeks) infiltrations. I've also played around with the uranyl acetate stain - 2% uranyl acetate in 70% ethanol for not too long (2-5 min) worked reasonably well on thickish (dark gold) sections of medium grade LR White. Rosemary White <rosemary.white@csiro.au> 09 Nov 2006

NETNOTES

SAMPLE PREPARATION – previously frozen tissues

I have to treat human stomach biopsies that had been frozen in liquid nitrogen and kept at -85ºC without any protectant. I have to fix and embed some of them for TEM and some for SEM. What should I do? I never had samples like these? I have a traditional ultramicrotome and no cryo devices. Annamaria Pisi <annamaria.pisi> 16 Oct 2006

If these were simply plunged into liquid nitrogen to freeze them, the ultrastructure will be virtually destroyed by ice crystal growth. You might check how they were frozen before you proceed to see if it's worth it. That said, you can freeze-substitute them then bring them to room temperature and embed as normal without cryo-devices. Get a small Styrofoam cooler (the kind chemicals are shipped in) and some dry ice and you are in business. A good substitution medium is 1% osmium tetroxide in dry acetone (dissolve from the crystals). Add about 1 ml of substitution medium to 2 ml cryovials, cap the vials and freeze them upright in liquid nitrogen. Transfer your samples to the top of the frozen substitution medium in a liquid nitrogen bath and loosely cap the vials, making sure there is no liquid nitrogen inside. For the substitution chamber, line a small Styrofoam cooler with some foil then put 2 beakers in, one inside the other. Add acetone to each beaker to about 1/3 full then chill everything by surrounding the beakers with dry ice. Put a few chunks in the acetone, too. Once it's all cooled add your samples with substitution medium then top up the cooler with dry ice and put the lid on. Usually a small cooler holds about 5 pounds of dry ice which will last 2 to 5 days depending on the outside temperature, but this should be enough to complete the substitution. Once the samples warm up to room temperature they are already fixed, dehydrated and ready to embed in the resin of your choice (or to critical point dry and put in the SEM). Don't forget to rinse a few times in pure acetone to remove the excess osmium. Kim Rensing <krensing@ucalgary.ca> 16 Oct 2006

SAMPLE PREPARATION - embedding acrylamide gel

I have a chemist who wants to look at nanoparticle distribution in fully hydrated acrylamide gel. Unfortunately the particles are too small to image using cryoFESEM. He wanted to just dump the stuff on a grid and put it in the TEM. After my emphatic "No", I finally persuaded him that it would have to be dehydrated, embedded and thin sectioned. Of course there will be the inevitable collapse of the gel when dehydrated. Does anyone have suggestions as to ways to fix the gel to try to stabilize it even a little bit prior to dehydration? Debby Sherman <dsherman@purdue.edu> 01 Dec 2006

Including tannic acid in the primary fix, following with uranyl acetate or OsO_4, has a good reputation for enhancing resistance of lattices against shrinkage. We have used the tannic acid-uranyl acetate sequence in acetone for freeze-substitution of muscle fibers, slam frozen while supported on a thin underlying block of 2.5% agar gel. (Nylon spacers limited squashing during the impact). The agar gel mesh appeared quite reasonably preserved when sections were oriented to include it; I can't say we took any pictures of it, because our interest was in the muscle. Mike Reedy <mike.reedy@cellbio.duke.edu> 01 Dec 2006

Polyacrylamide is one of the few plastics I have never found a way to section. Things I would try are: 1. lower the temperature to cryosection it (maybe below -100 C, which works for rubber) 2. embed the sample just like a biological tissue. It would be surprising if you saw much, anyway. Proteins in a gel will make a hairpin shape due to SDS binding. Knowledge of the sample would help you to know what to expect. Carol Heckman <heckman@bgnet.bgsu.edu> 02 Dec 2006

I quite share this opinion, however it really depends on the kind of information this person needs. I don't think the gel morphology is of interest, but more the nanoparticles themselves. In this case one does not have to care too much about what happens to the gel right? It is only supposition here, I am afraid. Perhaps one could just fix the gel as Paul described it and dry it with light heating and vacuum (like for protein gels). Perhaps it would be possible to get some interesting image in SEM (BSE should show a big contrast between the gel and the particles), but perhaps the resolution

would be an issue (and depth too). An alternative would be to embed the dried gel in resin (if it is possible, I have no idea but you'll know it only if you try it) and cut it for TEM observation. Stephane Nizets <nizets2@yahoo.com> 04 Dec 2006

SAMPLE PREPARATION – oil-in-water nanoemulsion

We have a investigator who would like to image an oil-in-water nanoemulsion. We have tried SEM and also applying it directly to carbon-coated grids, fixing with osmium, and viewing by TEM. The problem is that the nanoemulsion coalesces during processing. What would be the best technique to process and image this type of sample? Dotty Sorenson <dsoren@umich.edu> 27 Oct 2006

The best way to do this is cryoEM. Place a sample on e.g. a TEM grid, plunge into slush nitrogen (almost frozen LN_2), place on a cryostage, cryo-sputter coat, and into a low-voltage FESEM. CryoTEM might also work, but I've only done cryoSEM. Any method other than cryo can easily distort the emulsion droplets, and give inaccurate results. Phil Oshel <oshel1pe@cmich.edu> 27 Oct 2006

When I worked at a rubber company, we looked at synthetic latex by treating it with osmium and cryomicrotoming. A small plastic capillary tube held the treated sample in the microtome. This was frozen in the cryomicrotome. Thin sections were cut with a diamond knife and floated on icy cold 50/50 DSMO and water. The thin sections were picked up with a "perfect loop" and transfered to a carbon film grid. Of course the latex spheres were stable after hardening, I don't know about your oil. We were looking for hollow latex spheres, and we found a few. I was never convinced these rings were real and not some cutting artifact that dropped a section of core out of the hardened skin. Frank Karl <frank.karl@degussa.com> 27 Oct 2006

SAMPLE PREPARATION - cross section of multilayers on stainless substrate

I would like to know how to prepare cross section samples of multilayers on a stainless steel substrate. I have the basic knowledge of sample preparation of layers on silicon wafers using M-bond and ion thinning. However, stainless steel will be difficult to cut and grind. My sample is multilayers (such as Al2O3) on a stainless steel substrate, and the thickness of the multi-layers is around 3 μm. Each layer has thicknesses ranging from 10 to 1 μm. The other side of the multilayers is also stainless steel. The multi-layers are sandwiched by stainless steel. I will have 5 x 5 x 5 mm cubes. I will try to make TEM samples using ion-thinning, and later try to use FIB. Hiromi Konishi <hikonishi@gmail.com> 30 Oct 2006

What type of stainless steel are you preparing? If you have a martensitic stainless steel with any substantial hardness, you will have trouble. If you have an austenitic that is annealed, you will have less of a problem. I have a technique for preparing M50 and 440C substrates, but it is rather involved, so let me know. You have to prevent the sample from distorting because of the high internal stresses in the material. If you don't have the martensitic SS, then there are a number of ways that you can prepare your samples. One would be to make a stack, slice it, core drill the samples, dimple and then ion mill. Another choice would be to use the Tripod Polisher® technique followed by ion milling. And a third method would be to use the Technoorg-Linda method for preparing the samples. All of these have been used to prepare samples of coatings on metals. Scott Walck <walck@southbaytech.com> 30 Oct 2006

Don't know why you think stainless steel should be difficult to cut and grind - it's a lot easier than silicon! Have you considered spark erosion methods? Most (all?) steels can be cut readily by spark erosion. I would suggest that you follow a similar route to the one you are familiar with for preparing cross-sections from layers on Si wafer except: 1. When cutting the cylindrical core, use a similar trepanning tool but in a spark erosion system. 2. Having glued the core in to the Cu cylinder, slice by spark erosion with moving the wire. You then have 3 mm discs which are easily thinned further by mechanical grinding, if necessary, followed by dimple grinding and polishing. Final perforation can be achieved either by ion beam thinning or electropolishing - if your layers don't conduct, ion beam thinning is probably better. Larry Stoter <larry@cymru.freewire.co.uk> 30 Oct 2006

SAMPLE PREPARATION – negative staining with ammonium molybdate

I 'm making up a 5% ammonium molybdate / 1% trehalose w/v negative stain for a Special Project. Is there any trick to this? The solution is staying stubbornly milky so far. Does this clear up after doing a pH adjustment or is it just hard to get into solution? Randy Tindall <tindallr@missouri.edu> 20 Nov 2006

Why 5% ammonium molybdate? Way back when, I routinely used a 1% ammonium molybdate. If I remember correctly it did take a little while for the ammonium molybdate to dissolve, maybe overnight. The sugar may also interfere with the dissolving of the ammonium molybdate, but I have no idea about that aspect. Edward P. Calomeni <edward.calomeni@osumc.edu> 20 Nov 2006

I've never had a problem with molybdate. The milkiness suggests two things, pH or concentration. Several thoughts which may help. First, what is the pH you are adjusting to; ammonium molybdate is naturally weakly acidic. I can adjust the pH to 6.0 readily, but I have found high pH (>6.0) leads to poor solubility. Second, the concentration I use is 8.75mM, or just over 1%. This is adjusted so that the number of dense atoms is controlled between different stains – e.g. the number of Mo atoms able to deflect the beam at 8.75 mM is the same as the number of W in 2.5mM PTA or U in any formulation of 60 mM uranyl stain (acetate, oxylate, formate, sulfate, etc) You are using 5% - pretty high. Because the stain will dry down, do you really need that high of a concentration? Third, never used trehalose, but been tempted to give it a try. Does 1% trehalose go into solution readily? Does it affect the pH of the solution - perhaps raising it above 7.0? Will trehalose go into solution at lower pH? Can you adjust the pH to between 3.0 and 6.0, and if you do, what happens? Finally, when you mix the two compounds you will affect the saturation point for the two, and lower the relative solubility of each component. Solubility of ammonium molybdate is quite high. I don't know the solubility of trehalose, but Harris has used 10% solutions, and it's insoluble in organic solvents, and weakly soluble in alcohol. Perhaps you are affecting the saturation point of the trehalose by mixing with ammonium molybdate. I know Harris has done work as you describe, and believe Charles Humphrey at the CDC may have also, but I do not know if there are any side issues. I have tried mixed stains. Many times they reacted and caused all sorts of precipitates, colloidal milkiness, etc. Maybe they just won't work together under the conditions you are using. Paul R. Hazelton <paul_hazelton@umanitoba.ca> 20 Nov 2006

LM - dark field microscopy

What is dark field microscope? Pavitra Jain <pavi_micro@yahoo.com> 07 Nov 2006

A dark field scope produces an image of brightly illuminated objects on a dark background. It was one of the earliest attempts to increase the resolution and detectability of objects. Abbe's theory of resolution states that for good resolution you need to capture two of three possible rays. This is assuming that each microscopic object acts as a diffraction grating. This isn't too bad of an assumption. The three rays are one direct, undeviated ray from the sample and two primary diffracted rays. For simplicity we like to think of these thought experiments and the resulting ray diagrams as two dimensional drawing on a sheet of paper, because in reality is we are dealing with complex illumination issues. For some subjects the diffracted rays fall outside of the light accepting ability of the objective and these objects can not be resolved. One approach is to tilt or move the condenser off center. Many of the old medical grade scopes had this ability. This allows the undeviated ray and one diffracted ray to enter the front lens of

the objective. This produces a dark background and white illuminated subjects. A second approach is to use a central stop in the condenser to block the central ray and allow only highly angled rays to illuminate the subject. The diffracted rays from these angled light rays form the image. You often need a powerful light source or work in a very dark room with dark adapted eyes because you are throwing away a lot of light with a central stop. Special condensers were, and I believe, are still made to efficiently produce darkfield illumination. Newer illuminating systems, like phase contrast, have replaced a lot of darkfield work. Still some incredible images can be formed with darkfield. One easy

short cut is to use a phase contrast scope and select a condenser phase ring larger than the ring in your objective. Frank Karl <frank.karl@degussa.com> 07 Nov 2006

While Frank Karl is undoubtedly correct that the spur to invent dark field was based on resolution and tilting beams, I wanted to mention that there is a useful rubric for classifying types of microscopy, and that is based on contrast. Like this: Brightfield: Absorption contrast. Darkfield: Scattering contrast. Fluorescence: Fluorescence contrast. Phase: Optical path contrast. Nomarski: gradient in optical path contrast. Polarized light: birefringence (molecular alignment) contrast. In each case, the microscope takes advantage of a specific kind of interaction between light and matter to generate contrast Of course there are overlaps, e.g. scattering contributes to contrast in a brightfield microscope, etc. The Nomarski one may be hard to understand, this kind of optics gives rise to a signal whenever the gradient in optical path in a local region is different than what the gradient is in the background. Tobias Baskin <baskin@bio.umass.edu> 07 Nov 2006

LM - phase contrast vs. dark field

Could someone please explain the difference between phase contrast and dark field microcopy? What optic effects does one get with one or the other? I see Frank Karl states in his very nice explanation of dark field sent to the list the following: "One easy short cut is to use a phase contrast scope and select a condenser phase ring larger than the ring in your objective". Can phase contrast be transformed into darkfield then? Antonio M. Garcia <antoniomiguel.garcia@gmail.com> 10 Nov 2006

Darkfield allows you to detect objects by virtue of their ability to scatter light. Scattering is weak so you have to "get rid" of the non-scattered, incident light. The way you do this is to have the light incident at an angle that is too wide for the lens to gather. Imagine a hollow cone of light coming up through the condenser that is at an angle too wide for the numerical aperture (NA) of the objective. This hollow cone of light converges on the specimen and carries on past the objective. If the specimen is empty then looking down the tubes you see darkness. That is where darkfield gets its name. But if you have some scatterers, you will see light from them against the dark background, and the more they scatter the brighter they will be. Darkfield is great for picking up dust and debris, among other things. That's the concept. Now, how do you get a hollow cone of light? Well you can see that the difficulty depends on the NA. The higher the NA of your objective the trickier the condenser design needs to be to achieve an angle greater than the objective NA. So if you have a modest to low power objective, then a quick and easy way to get such a hollow cone is to use a phase ring designed for a high NA lens. Phase rings exactly provide hollow cones of light and by using a phase ring designed for a high NA objective (i.e., wide light cone) with a low power objective, the hollow cone of incident light passes that objective by and you get darkfield. Obviously, this approach won't work if you want to do darkfield with a high NA lens, and for that you need a specially designed darkfield condensor. Now, phase contrast allows you do detect objects by virtue of their optical thickness, which changes the phase of the light passing through them compared to light that does not pass through them. The tricky bit is to convert that into something your eye can see. This is done also with a hollow cone of light but in this case the cone is gathered by the objective and in the rear focal plane of the lens is placed a ring that lines up with the cone. The ring in the objective rear focal plane changes the phase of this incident light, relative any light that goes elsewhere through the rear focal plane of the objective. That is ultimately how the phase difference between light that goes through the sample (and hence elsewhere in the rear focal plane) and the background (light through the phase ring) is detected. The optics behind phase contrast are much trickier to grasp intuitively, indeed Zernicke won a Nobel prize for inventing it, whereas darkfield has been played with for ages. Tobias Baskin <baskin@bio.umass.edu> 10 Nov 2006

At the risk of creating another bump and run... Phase contrast and dark field are different. Phase contrast converts phase differences between the object of interest and the surrounding material to amplitude difference, which is what the human eye is sensitive to. One could think of darkfield as a way of increase detectability. Using a large phase ring in the condenser is a trick. Just a short cut to get illuminated objects on a dark background. I refer you to Tobias's excellent explanation. Frank Karl <frank.karl@degussa.com> 10 Nov 2006

I'll add another perspective on their descriptions: Dark Field: The cone of light that goes through the sample coming out of the condenser is more like a thin annulus. Think of it as a party hat (thin outer rim coming to a point). It is at a very high angle (outside the field of view). The angle that exits the sample layer is too high to be captured by the objective. Think of a party hat on top of another party hat point-to-point. The brim of the top party hat (that should get captured by the objective) is too big. Thus, when no sample is in the light path, the field of view is dark (darkfield). Small specs or the edges of samples (high change in phase) scatter the light so that it changes angle of the rays at that point and does get captured by the objective. One then sees white edges or spots (where this occurs in the sample) over a darkfield (where there is no scattering). Phase Contrast: In the most common use, the cone of light that goes through the sample coming out of the condenser is a broad annulus. Think of it as a party hat made out of very thick cardboard (outer rim coming to a point). It is at a moderate angle (inside the field of view). As the light passes through the specimen there are a) rays that are normally seen and do not touch any of the specimen and b) there are some that go through the specimen. The ones that go through the specimen are retarded (many biological specimens retard by 1/4 of a wavelength of light or within a wavelength range). As they come back through the objective, some encounter a dark (quasi opaque) annulus in the back focal plane that matches the optical size of the annulus of light from the condenser. This is called a phase ring. The phase ring set-up is of the correct thickness to either retard the rays by 1/4 of a wavelength (positive phase contrast) or to advance them (negative phase contrast). The rays going through the specimen are then doubly retarded - hopefully many by 1/4 plus 1/4 = 1/2 wavelength, while other rays are not. When they recombine, those rays that are 1/2 wavelength in difference (out of phase) cancel each other producing darkness. Those that are in phase combine to be bright. This dark (black) vs bright differences provide contrast - hence phase contrast. The actual retardation (phase shift) is dependent upon thickness and refractive indices. For positive phase contrast, because many rays do not get their phase shifted, the background is bright with objects or edges dark. For negative phase contrast, the background is dark and the objects are light. Let's take a phase annulus for phase contrast setup. Let's presume it was designed for an objective of higher numerical aperture (say NA = 0.85 for a 100x objective). If we use a 40x objective with an NA of 0.65, only the light scattered from the specimen will reach the objective. This will create a darkfield effect. It will not be the optimum though - only the proper sizing will do that. Another comment, because phase differences are related to thickness, one can see artificial repetitive lines (series or halos) from where thicknesses create phase shifts of 1/4 wavelength plus 1 wavelength, 1/4 wavelength plus 2 wavelengths, 1/4 wavelength plus 3 wavelengths, 1/4 wavelength plus 4 wavelengths, etc. One will also see some effects from 2nd and 3rd harmonics of the main constructive/destructive inference. And one more comment, because of the

phase shifting aspects enhancing edge effects, one can increase detection of Becke lines for refractive index matching of isotropic materials (glasses, for instance, in forensic applications). This can also be improved with Nomarski DIC under similar principles to an order magnification better than typical brightfield Tony Havics <ph2@sprynet.com>

TEM – digital cameras

We are a pathology/hospital based TEM facility equipped with a JEOL 1010 electron microscope. We would like to change to a digital camera system, since we find that wet-film technology is slow and expensive. I am hoping that there are users out there that can provide me with opinions about digital camera systems to help educate me about what they like, and what they think is important in a TEM digital camera and its associated computer [hardware/software] system. I have never used a TEM digital camera, so I have no experience as such with any digital camera system, other than consumer digital cameras, so it's hard for me to judge the merits of a digital camera without "getting my feet wet" so to speak, and trying it out. I'm open to suggestions, thoughts, and opinions of experienced users or any comments on this topic. Garry Burgess <gburgess@exchange.hsc.mb.ca> 12 Oct 2006

I'm sure you are going to get a number of answers to your question. As a manufacturer of these cameras, let me point out a few things that I think might help you. I don't want to make this into a "commercial" so I will stay away from specifics and try to provide some generic information that applies to all manufacturers. 1) TEM cameras come in two flavors: Bottom mounted and side-mounted. The bottom-mount cameras are in general better for high-resolution imaging, while the side-mount cameras give you a better field of view. However, I would not automatically dismiss the bottom-mount cameras, as there are ways to overcome the smaller field of view (automatic image montaging), and you might find that a bottom-mount camera is better for your needs. Bottom-mount cameras are typically more expensive, so your budget will play a significant role. 2) Throughput. Most pathology departments we have talked to put a premium on efficiency so that they can work on as many cases as possible per day. If that is the case for you also, the live speed of the camera is important. It is a lot faster if you can work with the camera in a live mode, then simply push a button to snap a picture, than to work on the microscope in the traditional way (binoculars), then switch to the camera (insert in beam or lift screen), then wait for a few seconds to take a picture. If the picture didn't work out, you would have to go back to the binoculars, adjust the microscope, and start over again. A reasonably fast camera (10 frames per second) lets you work on the computer screen directly. 3) Organization. Since digital images come cheap, users tend to take more pictures. If you have multiple users it can get quite busy on the hard disk, and you will have to implement some structure to keep users, cases, tissues, etc. apart. Here it might be advantageous to have a way of organizing your images in a database. If that is important to you, make sure that you are not missing out on that later. 4) Other instruments: Most labs have other instruments that are used for the same sample. For example, a grossing station for taking pictures of the tissue and a light microscope for color pictures. If you have that, it might be advantageous to select a system that allows information sharing and the same user interface between electron microscope and other instruments. 5) Hospital. Recently, there has been a push by pathologists to use workflow oriented software for their work, similar to what the radiologists have done for years. If this applies to you, make sure that you have an option to interface with HIS or PACS systems. 6) Reports: In most pathology labs, a technician acquires the images and hands them to the pathologist who then makes a diagnosis based on the images. This can be a critical step. Some pathologists are very particular how the images need to look like (contrast, brightness, magnification, even paper), others work with multiple formats. Whatever is the case, you need to anticipate this. If the pathologist insists on paper prints in a certain format, the printer becomes an important part of the system. It needs to be fast, and it has to have the right tint. That's not easy to find and you need to talk to the system manufacturers for help. You might also ask the vendors about report capabilities of their software. Predefined and customized reports make it a lot easier to have the "right" format for the pathologist. A digital system, however, allows you to get rid of the paper altogether. If you use a database for your images, you can probably install some software on the pathologist's desktop computer, they can then quickly find the images and make a diagnosis on the computer. If you think that this might be a possibility, ask the vendors about this option. 7) Support: Make sure that you can get good support for your system. Nothing is more frustrating than having to go back to film because you can't get the support you need. 8) Microscope tuning: For newer instruments, there is often an option for tuning the microscope automatically. This can help save time and make imaging easier for new staff. If that is important to you, find out if the software supports tuning the microscope. 9) Advanced features: If you require advanced features such as Tomography, make sure the system you buy supports it, or at least supports a file format that can be accepted by the third party software you use for the advanced features. In most pathology situations this is not of prime importance, though. Michael Bode <mike.bode@olympus-sis.com>

Crystallographic indices

I would like to know the tricks for getting Word to write a number with a bar over it (as is used in crystallographic indices). I believe that this has been the subject of past posts and I have tried to find them in the archive but perhaps I am not using the right search terms. My apologies for bringing it up again. I have had a solution that has served me well for a decade, but now I have a situation where it does not work. I recall that several solutions were proposed and I would like to try another. Help please. If you are interested, what I have been doing until now is to create the symbol (e.g. bar-one) with the equation editor and then making an "autotext" entry to insert it. Alwyn Eades <jae5@lehigh.edu> 10 Nov 2006

One possible solution is to install in Windows a special crystallography font (crystallography.ttf) available at: http://x-seed.net/freestuff.html. I hope this helps, Leszek Kepinski <l.kepinski@int.pan.wroc.pl> 10 Nov 2006

Word allows you to overwrite two characters in the same position by using one of the field code functions. To make "bar-1" you should: (1) press ctrl-F9 (this will automatically insert two curly brackets { } in which you can type the field-code instruction; (2) inside the curly brackets type EQ \o(1,¯) - you may need to play around to find the "overbar" symbol. On my system I can find this using Insert:Symbol (it is called the "Macron"). Alternatively you can try alt+0175 (using the number keypad). (3) press alt-F9 to display the text (you may need to do this twice). You can toggle between the field-code and the text using Alt-F9. I seem to remember reading somewhere that for some language settings (where the decimal separator is defined as the comma?) you may need to use a semi-colon instead of a comma to separate the two characters (i.e. (1;¯)). Hope this helps, Andy Godfrey <awgodfrey@mail.tsinghua.edu.cn> 10 Nov 2006

Using Andy's solution I created a small Word file that contains the glyphs 1 to 9 with overbars. I copy and paste these compound glyphs into any document that contains Miller indices, as needed. Great suggestion! … MT Editor

EBSD - sharpness

The sharpness of EBSD patterns decreases as the SEM kV is reduced. Does anybody know why? The only explanation I can come up with is stray fields. The lower kV electrons are going to be more affected by stray fields. Larry Stoter <larry@cymru.freewire.co.uk> 18 Oct 2006

I would suspect that either you are getting more contribution from the amorphous surface oxide layer as the beam penetrates less at lower kV, or that you are getting more contribution from the thin, deformed layer left over from polishing. The EBSP electrons come from very shallow in the specimen, because the ones from deeper in the sample don't come out with enough energy to contribute to the pattern, so the pattern is very sensitive

to the condition of the surface and top micron of the sample. Mary Mager <mager@interchange.ubc.ca> 18 Oct 2006

Isabell and Dravid have considered the problem in a paper on "Resolution and sensitivity of electron backscattered diffraction in a cold field emission gun SEM" in Ultramicroscopy 67 (1997) 59-68. Concerning the stray fields that you mention as a possible explanation they write: "Some high-resolution SEMs have a strong objective lens field which "spills" onto the specimen. This results in bending of Kikuchi lines due to the excessive magnetic field of the objective lens. This problem, however, can be easily solved by placing a small aperture of permalloy above the specimen, thus trapping the magnetic flux from the lens." More generally, they conclude that you get a lousy signal-to-noise ratio at low voltages because low energy backscattered electrons are much less efficient in exciting the phosphor than high energy BSEs. Hence the pattern quality is degraded at low voltages. Joergen Bilde-Soerensen <j.bilde@risoe.dk> 19 Oct 2006

The question was a general one, not related to a specific experiment/instrument, although it does seem to be a widespread observation. A few more thoughts: 1.The reduction of interation volume at lower kVs leading to a larger proportion of the signal coming from a less than perfect surface (or contaminating surface) seems the most likely explanation. Would electrochemical polishing or glancing-incidence argon ion milling provide a better finish than colloidal silica? 2. I don't think it is related to energy spread in the electron beam - there doesn't seem to be a difference between pattern sharpness on cold FEGs compared with Schottkey FEGs or W instruments. 3. While there might be distortion problems with SEMs which don't have fully contained fields, the loss of sharpness at lower kVs still occurs even on SEMs with fully contained fields and at longer working distances. 4. While I agree that the S/N ratio does deteriorate at the lowest kVs, the loss of sharpness is still apparent at kVs where this shouldn't be an issue. For example, the pattern sharpness does deteriorate in going from 30 kV to 10 kV. 5. Larry Stoter <larry@cymru.freewire.co.uk> 19 Oct 2006

You need to define "sharpness." What I think you are seeing is reduction of S/N ratio. The Kikuchi patterns are generated by a point source. Thus, moving the phosphor screen of the camera towards or away from the specimen will change the width of the patterns. Likewise, changing WD will also have a similar effect. Higher KV will provide deeper penetration of the specimen (~50 nm) and will produce good S/N. So it seems that what you are talking about is contrast or definition of lines. It is not "sharpness." Ideally, the PC should be about 1/3 down from the top of the scintillator disc. If you are off, that will degrade the pattern quality. Also be sure to set your analysis WD for the WD actually used. Gary Gaugler <gary@gaugler.com> 20 Oct 2006

I agree that there is a need to separate 'sharpness' from 'contrast'. And 'contrast' does relate to S/N and probe current. However, there does still seem to be a loss of sharpness at lower kV, as opposed to S/N. I think the likely explanation relates to surface damage and the smaller interaction volume at lower kV. Clearly, sample preparation is critical for good EBSPs - and this means a sequence of steps. Any individual process will generate its own surface 'damage'. The key is that each step should remove the damage from the preceding step while generating a significantly thinner damage layer. It is also necessary to understand the mechanical properties of the materials being prepared. My personal experience is with austenitic stainless steels, where mechanical processing can easily generate dense dislocation tangles (work hardening) several 100 μms deep. Polishing with colloidal silica or ion milling won't remove even half of the damage layer from the initial mechanical preparation - which is why I spent a lot of time with spark cutter systems. Larry Stoter <larry@cymru.freewire.co.uk> 20 Oct 2006

A couple of other things came to mind after my first posting. A real killer of patterns is oxidation. With EBSD data coming from only about 50nm deep, it does not take much to reach a point of no patterns at all. I baselined Al on Silicon wafers and after polishing (mechanical down to .02u colloidal silica) the patterns slowly diminished over three days after exposure to air. Then, in about an additional four days, no patterns. No doubt at all that specimen prep is critical to good patterns and even getting patterns at all. And indeed, different types of specimens require different prep methods. This is learned the hard way. Another factor is the binning value of the CCD. This works against lower KV in that the patterns can be very sharp without binning but the fps are very low. Like 3-5fps is not uncommon, but the images are great. This is mostly useful for collecting a single nice looking pattern for publication or reporting. Also, at lower KV you will need to increase camera gain (contrast). This tends to make the final pattern un-even from top to bottom. Doing an initial good adjustment of gain and brightness level along with background subtraction helps a lot in this respect. In some cases, adjusting WD will even-out the overall video signal. Another issue is whether the camera/phosphor is tilted or horizontal. Phosphor should be tilted up, camera down. This way, the incidence angle of the pattern for each point collected is symmetrical. I'm sure that the EBSD gurus can explain it better than I can. Gary Gaugler. <gary@gaugler.com> 20 Oct 2006

EBIC vs. SE

I'm puzzled by the interpretation and value of EBIC as specimen current monitor versus simple SE detection. If a specimen is imaged with E-T SE and then with specimen current monitor (SCM) via stage current monitor, what might the differences be? I see them as a measure of conductivity for SCM whereas the SE is generation of reflected SEs. Does the nature of reflected SEs directly correlate to the amount of SCM? What info can be gleaned by using SCM versus SE? All thoughts invited. Gary Gaugler <gary@gaugler.com> 21 Nov 2006

EBIC and specimen current are not the same thing. For specimen current imaging, you image the balance between the incident beam current (*Ib*), the current due to backscatters (Ibse), the current due to secondaries (Ise) and the specimen current (*Isc*), such that: *Ib=Ibse+Ise+Isc* Or, *Isc = Ib - Ibse - Ise* Thus, specimen current is like looking at the opposite of the backscatter and secondary signals. EBIC, on the other hand, is a semiconductor-specific technique. In specimen current, you put the current amplifier between the sample and ground. In EBIC, however, you put the current amplifier between the *p*- and *n*- sides of an electrically active diode. When beam electrons hit the sample, they can excite it in a way similar to light falling on a solar cell. The energy imparted by the beam causes current to flow through the external amplifier. EBIC then lets you image areas of high or low electron-hole-pair recombination activity in the imaged device. If you ground the *n*-side and operate above the unity yield point, you'll suppress most of the specimen current contribution and therefore most or all of the topography and get a purely semiconductor-device-physics signal. EBIC signals will often be a few orders of magnitude higher than the beam current, whereas specimen currents tend to be on the same order as the beam current. Chad Parish <chad.parish@gmail.com> 21 Nov 2006

I agree that EBIC is not SC. In my case, it is a matter of conduction rather than true EBIC. I'm not looking at junctions, etc. The idea is to measure and image using specimen current and compliment with SE and or BSE. I image with SC and mix with SE or BSE and get quite interesting results. In some cases, I'm using non-conductive specimens. In these cases, the beam current conductive portions have higher SC than areas that are non-conductive. However, in working with STEM specimens of IC cross sections, the metal conducts a certain amount while the passivation and ILD allows the beam to pass through. Depending on many factors, the SC can be radically different. I suppose I'm just thinking at the keyboard. But I do wonder what the physics is behind all of this practical application. Gary Gaugler <gary@gaugler.com> 21 Nov 2006

CORRECTION to Piekos, Diffracted Light Contrast ..., MicroscToday, November 2006, pg 10:

The magnification given in the legend to figure 2 is incorrect. Since the figure was reduced to single column width, the correct magnification should be 50,000x--not the 80,000x given.