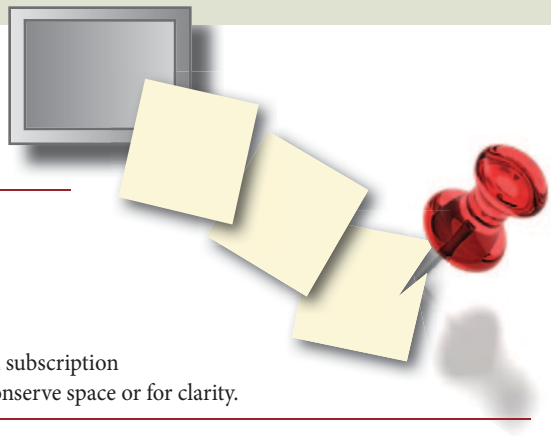

NetNotes

Edited by Thomas E. Phillips
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Specimen Preparation:
cleaving silicon wafers

One of our researchers is experimenting with photo-resist patterns on 6-inch silicon wafers. Our task is to image cross-sections of tiny little lines contained in rows of tiny little dots about the size of TEM grids. Imaging magnifications are around 100-200kX, so we're talking little here. Getting these wafers to break accurately is proving to be a real bear and it may be that the break is distorting the cross-sections. I'm using a diamond scribe to score the wafers as accurately as I can, but scribing the back of the wafer to get an accurate break on the front is proving to be difficult. There must be lots of industry folks out there who do this on a daily basis. Would someone be willing to share a few tiny little hints? ***Randy Tindall tindallr@missouri.edu Mon Feb 16***

I more than occasionally make cleaves through Si wafers, with several types of resist, with the intent of cross sectioning very small features for SEM. Typically these are scribed on the front side with a diamond scribe and cleaved with polymer glaziers-pliers. If I need to hit a very small (sub-micron) feature exactly, I make a series of reductional cleaves winding up with a SELA microcleaver to get the final cross section. Resist is one of the harder samples from which to get a good, undistorted specimen. Then you need to get an undistorted image. I don't cleave TEM samples. **John Heckman jwheckman@earthlink.net Mon Feb 16**

You may already be aware of this, but if not... All of the silicon wafers I work with, and cleave into pieces, have preferred cleavage planes along the crystal lattice. The directions and angles will depend on the type of wafer you have. The wafers I work with, which are for integrated circuit fabrication, have two very nice cleavage planes, at 90 degrees apart, which works nicely for cleaving cross sections of the circuitry. I can cleave a straight line across a 300 mm (12 inch) wafer by making a little scratch, maybe 1/8 inch long, in line with the cleavage plane, on the edge of the wafer. I then lay the scribe down on the bench top, with about 1/4 inch of the approximately 1/8 inch diameter shaft, under the edge of the wafer, lined up with the scratch mark. Then I hold one side of the wafer down to the bench top, and gently press the other side down over the scribe shaft. The crack starts at the scratch, and goes across the wafer. Occasionally, it will pick up a curve along the way, but most of the time it is as straight as an arrow. **Darrell Miles milesd@us.ibm.com Mon Feb 16**

Ask the researcher, or if necessary the wafer manufacturer, to tell you which crystal orientation is used in making the original wafers before they are etched, then scribe along a line appropriate to that angle. It makes a big difference; scribing and then breaking along the wrong angle shatters the wafer. I used to scribe part-way on the front of the wafer so that I could see where I was, and the break would continue past the score to give nice cross-sections - same idea as making glass knives for sectioning. **Lesley Weston leswes@shaw.ca Tue Feb 17**

Specimen Preparation
polymer film cross sections

Does anyone have a good technique for preparation of (cryo) cross sections of polymer films for SEM examination? I am interested in viewing the distribution of two polymers in a polymer blend. ***Wendy Cheng wendy.cheng@ipaper.com Wed Mar 25***

I've always just dropped the polymer samples in liquid nitrogen, grabbed and snapped. I did have one nylon sample that required repeated flexing before it would break, even at LN2 temperatures, but I still got good fractures for examining the cross-section. For thin films ... on paper? Snapping in LN2 worked there as well. If the films are separate, then you may need to clamp them between two thicker (but not thick) supporting pieces and snap the sandwich. This also works for thin films laminated (with bonding) between two thicker polymer pieces, although they will often delaminate (which can be useful). For films on substrates, it can make a difference whether the samples are snapped "toward" or "away" from the film, that is, whether the film is broken in compression ("toward") or tension ("away"). I can't give a generic one is better than the other, but I'd try breaking the film in tension first. Thick(ish) substrates may require cutting/scoring/sawing 1/2 way (or more) through first, from the side opposite the film. **Philip Oshel oshel1pe@cmich.edu Wed Mar 25**

If you are working in cryo mode inject the solution into a narrow drinking straw about 1/2 inch tall that is mounted in your cryo holder. Freeze the unit and then crack it open in your cryo manipulation chamber. I used glass rods for this in the early days of cryo but found that the very narrow drinking straws are far better. The best are those that are often used as stirrers for do it yourself coffee making! I think we are in the same area as a few weeks ago when we were talking about looking at cross sections but only if you could crack them in air? If the material will solidify in air, cast it onto a piece of aluminum foil. Once dry, place the foil in liquid nitrogen and then collect the media as it cracks off the foil. The different contraction rates of foil and media will cause the media to crack into nice cross sections. If the material is quite stiff or may be mounted firmly on a stiff surface cut it down to about 1 1/4inch by 3/8 inch. Place it in liquid nitrogen until the solution stops bubbling. Remove the specimen and bend it until it cracks. If it will not crack, neck the material at the half way point and try again. For more details and diagrams look at www.emcourses.com/crack.htm When a material will not fracture naturally and you are unable to use a cryo device, you need to stiffen the material. If the material is not soluble in water we use a water soluble carbon solution as the stiffener for example Agar G303. Drill a fine (1/8th inch) hole though two stubs placed face to face. Place your fibers/material through the hole and fill the additional space with the carbon solution. When dry plunge the unit into liquid nitrogen and when the liquid stops boiling take the unit out and crack it by striking the interface with a single edged blade. Take great care not to cut through the nit, simply crack it open. Once the two units are back at room temperature and the condensation has dispersed they are usable in the light microscope and the SEM. **Steve Chapman protrain@emcourses.com Wed Mar 25**

Specimen Preparation
PAS staining of epoxy sections

Has anyone ever stained with periodic acid – Schiff's (PAS) on Epon sections before? If not on Epon, then what type of plastic did you use to do a PAS stain? I know it is done on paraffin embedded material, but an investigator wants the PAS staining done on Epon sections? ***Anne-Marie Brun abrun@hsc.unt.edu Tue Feb 24***

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You would probably have to etch the resin to get PAS to work on Epon sections (see recent lister communications re epoxy resin etching methods). You might also consider using methenamine silver as an alternative to get a more strongly defined end result, particularly if you are using semi-thin sections of 2 microns or less. Alternatively if you were prepared to switch resin, PAS works on any acrylic resin but strongest with methyl methacrylate as you can easily remove the resin prior to staining. **Alastair McKinnon a.d.mckinnon@abdn.ac.uk Wed Feb 25**

http://treefrog.cvm.uiuc.edu/methods/PAS_EPOXY_CMI_UIUC.pdf **Lou Ann Miller lamiller@illinois.edu Wed Feb 25**

Sometimes it is desired to verify components in an epoxy section for PAS positive. Because the TEM epoxy blocks have ran the tissue through many different processes than the histology tissues go through, it is necessary to run the procedure differently, and understand the coloration of the final product may be different. The first problem is the hardness of the epoxy, stains, especially at room temperature do not always stain epoxy sections well. The second is that the fixative used in regular Transmission Electron Microscopy contains glutaraldehyde which will cause false positive staining if not dealt with. The use of saturated potassium hydroxide in methanol, used first off on the section to etch it, seems to take care of both of these issues. The tissue has also gone many reactions and exposures to heavy metals as well, osmium, and uranyl acetate in the processing. These chemicals change the tissue in a way that histology tissues are not; pretty bright magenta pinks are not seen. Instead a darker gray area is seen, in very thick places or sections, some of the pink may show through somewhat. It is because of this coloration, that for some instances, a counter stain is not used, especially the normal 0.5% toluidine blue with 1% Borate, as it will cover and obliterate the staining. Chemicals Used: saturated potassium hydroxide in methanol: 1 part KOH pellets, 2 parts methanol; let set 1-3 days before using, will have a remaining slurry of KOH on the bottom. 0.5 % periodic acid. Schiff's reagent - 1. Cut epoxy sections, for this procedure, 0.33 to 0.5 microns thick, and dry down well on a hotplate. Our hotplate is set at 120°. Dry at least one hour on the hotplate. 2. Remove slides and set the hotplate down to 60° to cool. 3. At room temperature, with the slide level, apply the saturated KOH-methanol mixture and allow to incubate for 20-40 minutes. 4. Rinse off gently with water, and allow to sit in water rinse baths for at least 10-15 minutes. 5. Air dry off the slide gently (hose to air outlet with filter on end) and place on the 60° set hotplate. 6. Immediately flood the slide with 0.5% periodic acid. Let set for 40 minutes, periodically checking to be sure the slide does not evaporate the solution away. Add more solution as needed. 7. Rinse off gently with water, and allow to sit in water rinse baths for at least 10-15 minutes. 8. Air dry off the slide gently (hose to air outlet with filter on end) and place on the 60° set hotplate. 9. Immediately flood the slide with the standard Schiff's reagent. Allow to stay on the hotplate for 40-60 minutes, again checking that the solution does not evaporate and adding more solution as necessary. 10. Rinse with water well, and soak in water baths for up to 15 minutes. 11. Dry slide and observe. References: Hayat, M.A., Stains and Cytochemical Methods 94, Plenum Press, New York, 1993, p 64. **Lou Ann Miller lamiller@illinois.edu Wed Feb 25**

Here is the very straightforward protocol I finally used on my Epon sections of intestine and it worked, although the staining was not very intense. I didn't really spend much time to improve the method though. - Periodic acid 5%: 30 min at 50°C - Schiff Reagent: 30 min at 50°C - Post-staining: Azur II mix (it is a 1:1 mix of methylene blue and Azur II, more stable than methylene alone): 20 min RT (section thickness: 300 nm) Additionally, one person told me he stained the glycogen using reduced osmium. Another one gave me this reference: Shroeder et al. (1980) "An established routine method for differential staining of epoxy-embedded tissue sections" Microscopia Acta 83,111-116 **Stéphane Nizets nizets2@yahoo.com Tue Mar 3**

Specimen Preparation

glycogen in TEM

We have a researcher who is studying the effects of a particular drug on rat liver, heart and spinal cord. Tissue for EM was processed with osmium tetroxide, en-bloc stained with uranyl acetate, dehydrated with alcohols and embedded in Procure 812 epoxy resin. Thin sections were stained with lead citrate. By EM we found increased amounts of glycogen in one of the liver samples. The researcher asked "How do you know its glycogen". I said "It just is". He is worried that his supervisor won't accept that it's glycogen just because we said so. He wants to prove it. Is there a stain for epoxy resin sections that will stain specifically for glycogen? Will periodic acid Schiff (PAS) work on resin-embedded sections? ***John Brealey john.brealey@imvs.sa.gov.au Tue Mar 24***

There is Thiery's silver proteinate method for glycogen staining in ultrathin sections on EM grid: Thiery, J.-P. 1967. Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. J. Microsc. 6:987-1018. We have used it with success to proof glycogen-like polysaccharides in Streptomyces long time ago, (1996). **Oldrich Benada benada@biomed.cas.cz Tue Mar 24**

Wanted to add two comments to discussion of retaining and identifying glycogen in tissue. 1) Several years ago there was a thread on the list about glycogen with primary input from Krystyna Rybicka. She maintained that the protein component associated with glycogen was subsequently washed out in dehydration if tissue was subjected to a pH change (ie. en bloc staining with UA) during processing. The remaining free-floating glycogen could then clump in the cell. She recommended avoiding that pH change during processing to retain the classic rosette structure. Once embedded, the PAS technique could be used for detection of glycogen on the LM level and the Thiery technique (which I think Wolfgang Muss mentioned) could be used for EM. Here are two references: a) K.K. Rybicka. 1996. Tissue & Cell 28 (3) 253-267. b) Microscopy Today, October 1994. "Glycogen Granules Revisited". 2) Many years ago when I worked at the Dana-Farber Cancer Institute we found that the following protocol was very useful in preserving glycogen rosettes in liver biopsies of pediatric cancer patients. It involved fixing the tissue with osmium potassium ferrocyanide (OPF). The recipe was: 2 ml 2% aqueous osmium; 2 ml buffer (i.e. 0.1M Na-phosphate, pH 7.4); 0.06 g potassium ferrocyanide. The OPF solution was prepared with thorough mixing just prior to use and administered to tissue for two hours in refrigerator. The general processing protocol was glutaraldehyde and OPF fixations, alcohol and propylene oxide dehydrations, and Epon embedment. We post-stained with uranyl acetate and Reynolds lead citrate. Uranyl acetate en bloc staining is not recommended. So I guess there are two important issues: preservation of glycogen in the protein-bound form and post-embedding identification by the Thiery technique, for example. I would think that the observance of rosettes is strong enough evidence. **Don Donald Gantz gantz@bu.edu Wed Mar 25**

Immunocytochemistry

storing slides

How long can slides with sections be stored before they are used for immunolabeling? 6 months in the refrigerator? Or longer? Or is it a bad idea to wait? I usually cut sections then label the next day but someone here would like to store the slides for awhile if that is an okay practice. The tissue is embedded in LR White. ***Beth Richardson beth@plantbio.uga.edu Tue Apr 7***

I store resin and paraffin for years with no apparent problem. I have stored cryo for months at -80°C but you risk desiccation or other problems. Usually it is obvious when the cryo morphology has deteriorated. **Thomas E. Phillips phillipst@missouri.edu Tue Apr 7**

Tissue should be relatively stable in epoxy even at room temperature. Consider Kristina Micheva's (Stephen Smith Lab) Array Tomography technique where LR White sections are repeatedly immunolabeled and each label is eluted with 0.2M NaOH and 0.1% SDS before the next label is

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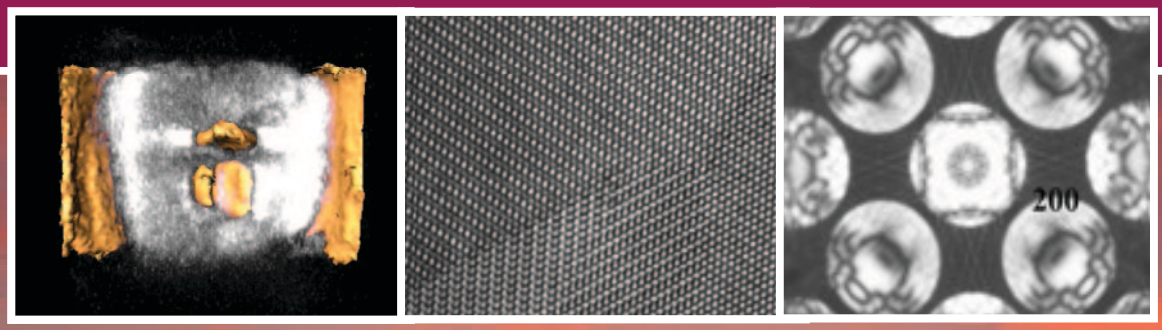

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applied. Of course, there is always an antigen that is the exception. **Larry Ackerman larry.ackerman@ucsf.edu Tue Apr 7**

When I was performing EM immunostaining in industry, we always assumed that tissue blocks in resin were preserved, but as soon as you section the block, the immediate block face and the sections will undergo changes as a result of being exposed to air and light (where applicable). In histology, some people will immunostain paraffin embedded slides that are many months old, but our careful time-course comparisons revealed that although immunostaining older slides produced a staining product, the sensitivity was greatly reduced after the first 24-36 hours, and further degradation occurred some 1-2 weeks later. Many antibodies worked fine after a month or two. I believe that similar results were observed with resin embedding, but I can't remember if I actually read a paper on it, or if we extrapolated the conclusion from histology IHC results. Someone must have published results on this, don't you think? **Gregg Sobocinski greggps@umich.edu Wed Apr 8**

I had always assumed that, as you say, the best immuno results were from fresh sections. I further understood that resin-embedded sections were fairly impervious to at least some immuno stains (especially of course immuno-gold) and so only the exposed cut surface of the section would present antigens to the label. If the fewer available antigens in a stored resin section are greatly reduced, then I would assume that staining might be more affected than a de-waxed section (or possibly a partially preserved cryo-section). **Malcolm Haswell malcolm.haswell@sunderland.ac.uk Wed Apr 8**

Now that I think about it, one of the trickiest variables I've observed when immunolabeling is the high degree of variability of antigen robustness. Some antibodies show consistent immunostaining patterns for years on sectioned paraffin slides, and are not sensitive to fixation variables. Others, especially recent proliferation protein antibodies and some other "high tech" antibodies, show high immunostaining variability depending on: 1) the fixative type, 2) the timing of fixation (sensitivity to over and under fixation), and 3) the tissue section age (as queried in this original topic). Yet another variable is the introduction and refinement of new antigen retrieval/unmasking techniques over the past fifteen years or so. Yes, the antibodies do not penetrate beyond the cut surface of the resin, unless the resin is reduced with sodium ethoxide. I agree with you that conserving the few antigen binding sites exposed on the EM section should be first priority, which is why I assume most people use fresh sections. I haven't been able to find any publications to share at this time. My apologies for the delayed response, as I mistakenly thought I'd have time to perform a literature search. **Gregg Sobocinski greggps@umich.edu Tue Apr 14**

Instrumentation

safety concerns with nitrogen supply

In response to a query on the safety of nitrogen gas supply next to a microscope setup:

There should be some sort of risk assessment (in UK law). I assume (but I'm not familiar with the apparatus) that there could be a potential for nitrogen venting out of the apparatus or supply lines. If there is, then one thing to look at is the potential for dropping the overall oxygen content by 1 or 2% in the room - you will need to check those figures. But, if it can happen, then an oxygen depletion alarm would be very important as a first step. Hopefully this will keep you happy too, because people do die from oxygen depletion because there is no warning. **Malcolm Haswell malcolm.haswell@sunderland.ac.uk Thu Mar 12**

You should also not discount the possibility of a pressure relief valve failure or poor insulation causing higher than normal boil off. I once had a tank's high pressure safety go off. Sounded like a jet plane and it filled the room with nitrogen gas in a matter of minutes. Called the fire department and they advised us to evacuate the room and go outside. They came screaming up with full breathing apparatus and checked the room for us before we went back in. No harm done, but pretty exciting. **Jonathan Krupp jkrupp@deltacollege.edu Thu Mar 12**

Sometimes good intentions go awry as well. Some years ago when a freezer or Dewar failed, a lab person stored items in a portable Dewar and then placed it in a cold room. It's cold in there and should last longer--right? Later someone else entered the room and nearly passed out due to the lack of oxygen. He was smart and had experience at high altitudes so managed to get out and survive. Whew! **Larry Ackerman larry.ackerman@ucsf.edu Thu Mar 12**

At one of CSIRO's labs in Geelong (near Melbourne), a CSIRO staff member died because none of the 3 safety mechanisms in the basement holding the LN2 freezers was working properly. He stepped down into the basement, passed out immediately and that was that - this happened about 8-9 years ago. Since then, the organization has been extremely careful about LN2, and we are no longer allowed to store large volumes inside. OK, it's a pain, but you can see where they're coming from. Only takes a couple of people to get annoyed with a beeping O2 sensor (this happens to ours when it's time to replace the sensor) and turn it off and you've got a recipe for disaster. Logically, all the safety stuff we have in our lab is completely over the top, but the answer to that is - we can't allow another accident like this one. **Rosemary White rosemary.white@csiro.au Thu Mar 12**

We have our LN2 filling station in a smallish room off our loading dock with an O2 sensor/alarm. With the doors open and a fan moving the cold N2 vapors out off the floor, O2 levels will drop from 20.8% (by the meter read-out) to 20.1% when filling a cold 10 L Dewar. **Richard Harris rjharris@uwo.ca Fri Mar 13**

As far as I remember, one liter of liquid nitrogen produces about 700 liters of gaseous nitrogen, which then doesn't contain any oxygen. In addition, the nitrogen vapor is cold, and will sink to the floor. If you experience a sudden drop in oxygen concentration, you may lose conscience, even if the relative oxygen concentration was still within the breathable ranges. If you then fall to the ground, you are in the nitrogen vapor, where you will not get any oxygen. If you stay there for 2 minutes without immediate help, you are gone. The minimum required O2 level for survival are somewhere around 10% (?). Normal values are 22%. I heard the rumors of an accident, where a graduate student in Germany tried to refill a LN2 Dewar in a cold-room (low ventilation) from another Dewar one Saturday. He overfilled, the LN2 spilled onto the floor, and he lost conscience. Another graduate student saw that, tried to reanimate his fellow in the coldroom, and both were found dead on Monday morning. Portable O2 meters can be found here: http://www.ceainstr.com/pdf_datasheets/gasman2_Info.pdf. These are portable devices with a digital display of the oxygen concentration. They are about the size of a calculator, powered by three AA batteries. They cost $614 per device, with about 12 months of life time of the oxygen sensor, and $110 replacement costs for the oxygen sensor alone. I have no affiliation with that company what so ever, except that we have a few of these gas meters. **Henning Stahlberg hstahlberg@ucdavis.edu Thu Mar 12**

I'm not certain of the official guidelines for symptoms, but they may be similar to asthma, as it's also a condition of low O2 when flared. Blacking out, or vision seeming to de-pixil some. Closing one's eyes after seeing a contrasted object, and still retaining the image for longer than normal when the eyes are closed. Slurred speech & slowed thinking; sometimes some dyslexia (when I start mixing words it's time for the inhaler). Sometimes even stumbling on your own feet. Fatigue, that goes away when you are not near the trigger Nausea, vertigo. **Lou Ann Miller lamiller@illinois.edu Sun Mar 15**

Large Dewars of liquid nitrogen should never ride an elevator with people in it. On the UC Berkeley campus and at the Lawrence Berkeley Lab, large LN2 Dewars ride the elevator alone with a large sign warning not to get on with it. The researcher takes a different elevator or runs the stairs. **Roseann Csencsits rcsencsits@lbl.gov Tue Mar 17** MT