NETNOTES

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Selected postings from the Microscopy Listserver (http://microscopy.com) from 2/14/06 to 4/14/06. Postings may have been edited to conserve space or for clarity.

LM - Floaters

A colleague asked me why she is so much more aware of the floaters in her eyes when she is using the microscope (or the telescope) than at other times. I assured her that it wasn't just her, it happens to a lot of us. But why floaters are so much more noticeable when looking in the microscope? Robert Bagnell, Jr. <bagnell@med.unc.edu> 17 Feb 2006

It has to do with the physics of simple pinhole optics. Essentially, when you are just in the right focal plane, you are doing an "entopic exam" of your eye. You can also reproduce the experiment by putting a small hole (about 1/8") into a piece of cardboard (1/2 of a file folder) and staring through it at a neutral surface (blank wall; sky, if not too bright). Adjust the distance between the card and your eye and, at the right distance, you will see the internal structure. I've had an interesting array of tiny cataracts for over 25 years and keep track of their position and size using this method. Barbara Foster <bfoster@mme1.com> 17 Feb 2006

SAMPLE PREPARATION - viral particles

One of my users has problems getting a good grid with negatively stained viral particles. She floats the grid on the virus + stain droplet, picks up the grid and then dries by touching it against filter paper. It sounds like a pretty standard procedure but she often found that the support film ("store-bought" carbon coated Formvar on either 300 or 400 mesh copper grid) is broken after the procedure. And occasionally, almost every hole is torn. Are there any tricks to prevent this? I have experienced similar broken films, but it was in Formvar coated slot grids. After picking up a group of ribbons, the support film broke when the grid is dried. I always thought that I was just careless during the handling causing the film to crack and eventually the surface tension tore the film completely. However, that would be unlikely for the 300 or 400 mesh grids that are supported by so many grid bars? Wai Pang Chan <wpchan@u.washington.edu> 01 Mar 2006

I have usually found that coatings collapse when the virus still has a lot of extraneous material associated with it such as from fecal samples or a not very pure cell pellet. I assumed it was mainly a heating and charging effect because of the high levels of background organic material which will swamp the conducting capacity of the carbon and copper on the grid. You never normally see it on pure isolates such as T4 phage. The simplest answer might be to dilute the drop until the grid stops breaking or spin out as much of the background as possible. Your slot grid problem can easily happen because of flexing of the grid, but it may also be weakened, if you just coat the flat slot grids with plastic. I usually bend them slightly upwards so the plastic stretched across the slot can't be damaged when it dries out. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 02 Mar 2006

When you say that she dries the grids by touching them to filter paper....is she actually touching the face of the grids, or wicking the excess fluid by touching the edge of the grid to the paper? This is a critical difference. It is all in the interpretation of a written protocol. Leona Cohen-Gould <lcgould@med.cornell.edu> 02 Mar 2006

Instead of floating the filmed grid on the virus/stain mix try attaching the grid to a grid stick that has adhesive applied. Or you can put a piece of double-sided scotch tape onto the edge of a glass microscope slide and attach the extreme edge of the grid to that. Now apply the virus/stain drop and after the appropriate time remove the liquid by touching the edge of the grid with a piece of filter paper. This provides much gentler handling of the support film than lifting the grid off a droplet of solution where the surface tension creates quite a pull on the film as the grid is lifted away. Certainly, using this technique, the support film should not rupture even on a 200 mesh grid. Ted Dunn <drteddunne@yahoo.com> 02 Mar 2006

SAMPLE PREPARATION - Cell culture preparation

We need advice on TEM sample preparation for cultured cells. We are growing skin fibroblasts in culture and would like to examine the cells with transmission electron microscopy. To date, our results have been disappointing. We grew the cells on Thermanox coverslips, fixed for 1 hr at room temp. in cacodylate-buffered 1.5% glutaraldehyde and 1.5% paraformaldehyde. After buffer washes, the cells were post-fixed with 1% osmium tetroxide, then washed, dehydrated in acetone and embedded in Epon/Araldite. The cells look OK in general, but the membranes (both plasma membrane and internal membranes) are all rather fuzzy and indistinct. The quality of the ultrastructure is much poorer than we obtain with tissues prepared using almost identical procedures. One would think that since the cells are only a monolayer, preservation would be excellent. If you have experience with TEM of cultured cells and have obtained good results, I would appreciate any advice you can give me to get better quality ultrastructure. Randy Tindall <tindallr@missouri.edu> 20 Feb 2006

Many years ago I fixed cultures of retinal pigment epithelial cells for TEM. I used a routine cacodylate-buffered glutaraldehyde fix, no formaldehyde although that should not matter. I did put 1% tannic acid in the fix, both glutaraldehyde and osmium and it helped show the ECM but I don't think it should be essential. I grew the cells in ordinary culture dishes, no Thermanox coverslips and embedded in Epon substitute. My guess is that your membranes look fuzzy due to less than optimal fixation of lipids (old glutaraldehyde and/or osmium?) Or extraction of lipids (too long in dehydration?). Why are you using acetone and not ethanol? It is my understanding that acetone removes lipids faster than ethanol but I have also heard that the converse it true. Certainly too long in dehydration can degrade ultrastructure. Also, add 2 mM Ca^{2+} ions to the fix to help stabilize the lipids. Geoff McAuliff <mcauliff@umdnj.edu> 20 Feb 2006

I have heard other people report situation like this and I, too, have experienced the same thing. Often time (not always) monolayer cells showed very little membrane contrast, even though other tissue processed the same way had no problem. The problem with monolayer cells seemed random regardless of what types of cells were being processed. More interestingly, I have not seen this problem with cell suspensions. In the past, I tried to use freshly made OsO_4 once when I had low membrane contrast problem with monolayer cells, and that helped. But I still do not understand why the problem only occurs in monolayer cells. I do not think it is a reagent penetration issue, nor a problem of inadequate processing protocol. Is it possible that some kind of coating material used in the culture makes it harder for OsO_4 to react with lipid molecules? Hong Emory <hyi@emory.edu> 21 Feb 2006

I process monolayers of cells frequently for TEM. I typically grow my cells on carbon coated, glow discharged coverslips. The problem of low membrane contrast is due to the lipids being extracted away during dehydration and embedding. Even membranes "stabilized" with OsO_4 can be extracted with the long processing times used during "standard fixations". I typically dehydrate in ethanol for 1-2 minutes per ethanol grade, with my samples dehydrated from 100% water to 100% ethanol in 15-20 minutes. I also keep the time in liquid resin to a minimum. My whole embedding protocol takes less than 3 hours. After I shortened my times considerably, my membranes started looking very nice and crisp! Here is my embedding schedule: When embedding monolayers (~60 - 90% confluent) in epoxy resin (I prefer Quetol 651) I follow this schedule: graded series of ethanol, 25%, 50%, 70%, 95%, 100%, 100%, total dehydration time 15-20'; 100% ethanol:Quetol 651 (1:1), ~25'; 100% Quetol 651, ~50'; 100% Quetol 651, ~100'; fresh 100% Quetol 651, then into a 60°C oven for polymerization. I do my processing in a 6 or 12 well plate that sits on a shelf in the hood and not on a rotator. I transfer my coverslips to a new 6 or 12 well plate

between changes of 100% ethanol. I also use minimal volumes of resin to decrease extraction of lipid components. If you use a more viscous resin, times may need to be lengthened. Eugene W. A. Krueger <krueger.eugene@mayo.edu> 21 Feb 2006

SAMPLE PREPARATION - propylene oxide

I was told by a technician of a certain company that acetone can act as a "scavenger" when used as a transitional solvent for Araldite 502/Embed-812 medium and propylene oxide would always be better. Why would this be? MM <martimor@nmsu.edu> 09 Mar 2006

I learned EM from John Luft around the time he pioneered use of Epon and propylene oxide. Four years later I diverted to acetone after being surprised and impressed by the results of Robertson, Bodenheimer and Stage (1963) J Cell Biol 19:159. Still later, I tested a 60°C heat-cure of 10 ml test-tubes filled with my Araldite 506 embedding mixture deliberately adulterated by inclusion of 15-20% solvent, trying propylene oxide, acetone, ethanol and methanol. I found no reason then or since to give up acetone in favor of the others. Specifically, the propylene oxide mix cured to give a softer and somewhat cheesy polymer; the acetone mix reduced somewhat in volume during cure and the cured product was similar to unadulterated resin. I can't recall the alcohols results; I think they were similar to acetone. I was surprised that the propylene oxide result was inferior to the acetone result; I had expected both to evaporate during cure to leave a final polymer unaltered by the solvent inclusion. I never heard the term "scavenger" applied. Luft called propylene oxide a "reactive diluent", and suggested that any small amount that failed to evaporate during heat-cure would incorporate harmlessly in the polymer. He was probably correct. My point is that large amounts of propylene oxide are not so "harmless", while similarly large amounts of included acetone are surprisingly innocuous. Mike Reedy <mike.reedy@cellbio.duke.edu> 10 Mar 2006

SAMPLE PREPARATION - MgO preparation and Fe oxidation

I have three problems. 1. Single crystal MgO preparation: I need a very good quality cross section TEM sample. I now manually polish it to 70 µm and dimple to about 20 µm and then ion milling (PIPS). But it's not so good. The edge looks sharp and MgO cracks a lot when I grind to less than 60 µm on diamond papers. Do you have better ideas to get a very thin specimen? 2. Another problem is Fe oxidation on top of the MgO substrate. It seems after some time, the sample has oxidized a lot. Even after I got a very thin specimen, it looks amorphous; it should be crystalline. I use plasma cleaning but it is not working very well. 3. Beam sensitivity of the MgO sample—how do I get rid of this? Chao Wang <chao.wang@materials.ox.ac.uk> 04 Apr 2006

I too have experienced similar problems in preparing thin specimens from single crystal MgO. I have had some success with plan view specimens, i.e. ion milling away the substrate to perforate through into a thin oxide film grown on it (see 2005 J Cryst Growth 285:208-214). Here samples were ground to about 80 µm thick, then dimpled. I never get too adventurous with dimpling - aiming for a thickness of 30 µm. Any thinner and the MgO has a strong inclination to fracture. All grinding and dimpling is done very gently to avoid cracking the substrate. I then ion mill in a PIPs at 5keV, 6 deg, until the sample is near perforation, then reduce the angle to 4 deg and the voltage to 3keV and then mill to electron transparency. To liberate the specimen from the mounting post I soak it in acetone till it drops off rather than heating to soften the crystal bond and slide it off —the latter is guaranteed to break thin regions off. Tripod Polishing was of no use at all, aside from the coarse grinding step to get down to about 80µms. Success was very hit and miss. I suspect my MgO crystals had a high degree of residual stress. Cross sectioning is even harder and requires a lot of patience to get any kind of result. Specimens just disintegrate in the PIPS without any mechanical handling. Focused ion beam milling may help if you have access to one. With regard to oxidation of Fe, my experience is with electropolished foils rather than thin films, but there are some similarities. Storage in solvents like methanol is definitely not recommended—such polar solvents are highly corrosive to iron. Storage in a non-polar solvent like a hydrocarbon may stop oxidation, but cleaning

it up for TEM examination could be a challenge without a plasma cleaner. Storage in vacuum is surprisingly bad for electropolished foils and they oxidize much more severely than storage in a normal lab desiccator. I suspect the protective hydrated film formed from electropolishing dries out in vacuum and cracks allowing oxidation to proceed. Of course your films don't have such a layer, so vacuum storage may be no worse or better than a good lab desiccator. Probably the key factor governing oxidation is the level of water vapor your specimen gets exposed to. With respect to beam sensitivity - I presume you mean charging rather than beam damage? At 200keV I did not experience significant damage in the MgO substrate, but electron beam charging was a major issue. I therefore always evaporate a very thin layer of carbon (20Å) onto MgO specimens prior to TEM examination. If the film grown on the MgO is not very conductive, then I coat both sides. In your case you have a conductive layer on one side (Fe), so you may get away with coating just the MgO side. I hope this helps. Dave Mitchell <drm@ansto.gov.au> 04 Apr 2006

I don't quite understand the question you are asking about specimen preparation—you say "cross-section", but cross section of what? However, I do know that over a period of (many) months, MgO samples transform into what appears to be $MgCO_3$. For a period of years, I had a student working on polycystalline MgO. He made samples by ion milling. He did not have to take special precautions with his samples if he examined them days or weeks after they were made, but if we wanted to look at them again a year or two later we had to "tickle" them with the ion mill to clean them up. STEM analysis showed that the carbonate layer had formed. We did not try to prevent this happening as it was not a major problem. MgO is known to be beam sensitive. A significant part of the damage depends on the current density, so working in a FEG-STEM you can be much worse-off than imaging or using a less intense electron probe. But you can't eliminate the problem. Going to lower bean voltages can (counter-intuitively) make the problem worse, as the cross-section for various electron-sample interactions can actually increase at lower voltages—though the total current in the electron probe will also decrease, which may partially or totally compensate. Tony Garratt-Reed <tonygr@mit.edu> 04 Apr 2006

IMMUNOCYTOCHEMISTRY - nanogold in semi-thin sections

Using pre-embedding histochemistry, a client has infiltrated lung tissue with gold nanoparticles of various sizes. He would like to see the distribution of the particles on semi-thin sections and then examine the tissue with TEM. Does anybody out there have a good protocol for silver-intensifying the gold on 1 micron Epon sections for light microscopy? Any suggestions would be greatly appreciated. Ralph Common <rcommon@msu.edu> 15 Mar 2006

Enhancement for LM has been done for decades, both with published recipes (Danscher, Burry, Bienz and Springall and Lackie from the Hammersmith EM research group in London) as well as with commercial reagents. There are a number of companies who produce silver (and gold) enhancement reagents for light and for electron microscopy. If the particles are sufficiently enhanced you will be able to pick up individual particles in the light microscope bright field image, and certainly in epi-polarization mode. In our experience, enhancement will mostly be limited to particles on or close to the surface: they have to be exposed to become enhanced. Depending on the resin, there seems to be some penetration with larger particles on the surface vs. smaller ones below the surface. To visualize the particles in LM the particles need to be relatively big, and visualizing the same specimen in EM might not be ideal. But I guess your client wants to check the specimens in LM and if a signal is found, look at unenhanced ones in EM? I initially (probably mistakenly) assumed the study was about discriminating between particle sizes after enhancement. Even though the size of the enhanced particles will somewhat depend on the initial size of the gold particles, I seriously doubt it would be possible to discriminate between sizes using LM techniques. In fact that may even be hard in electron microscopy, unless the initial particles were significantly different in size. On the other hand, double labeling using silver enhancement and ultra small gold particles has been successfully done in pre-embedding EM (Yi, H., J. L.M. Leunissen, G.-M. Shi, C.-A. Gutekunst, and S. M. Hersch. A Novel Procedure for Pre-embedding Double Immunogold-Silver Labeling at the Ultrastructural Level; J. Histochem. Cytochem., March 1, 2001; 49(3): 279 - 284) Jan Leunissen <leunissen@aurion.nl> 16 Mar 2006

MICROTOMY - section thickness

Can anybody provide an explanation concerning the refractive index dependency of the interference colors seen in charts for estimating the thickness of ultrathin sections? The small print in such charts usually reads "valid for refractive index of ca. 1.5", which is fine for methacrylates and similar embedding media, but what type of shift (if significant) would be observed for a lower refractive index, say 1.3? I am not sure whether the usual charts are valid for the interference colors given by cryosections. Andrew Leis <leis@biochem.mpg.de> 17 Feb 2006

As I understand it, the colors seen in thin sections are caused by destructive interference. The equation: Wavelength = $2nt/m$ describes the color seen. n = refractive index of film. t is thickness (nm), m is any integer. If so, it seems that as refractive index decreases, wavelength will also decrease for the same thickness film. Frank Karl <frank.karl@degussa.com> 21 Feb 2006

MICROTOMY – cleaning grids

Since about a month we have been having a problem keeping the sections stuck to the grids. The grids are cleaned in 100% acetone and dried. Sections are picked up either from above or below the water. When the grids are stained using the microwave staining method we have been using for several years the sections come off the grids. Every now and then the sections will stick to the grids and everything is fine. I have tried staining less time in the microwave, but this does not make a difference. Are there different ways to clean the grids so the sections will stick better? Eric <biology@ucla.edu> 15 Mar 2006

Just before picking up sections dip the grid into 1N HCl, then in acetone and in beaker with distilled water. Vladimir M. Dusevich <dusevichv@umkc.edu 15 Mar 2006

Some brands of grids are routinely treated to prevent surface oxidation. If that treatment was not carried out properly on a particular batch of grids then sections and support films tend not to stick to the grids. So long as its presence is not a problem for any reason you can dip the grids in a solution of poly-L-lysine which, when dry, helps the adhesion of sections. Poly-L-lysine is available from most EM supplies vendors. Ted Dunn <drteddunne@yahoo.com> 15 Mar 2006

EM - microscope cooling lines

For years we have been wondering where the dark material was coming from that accumulated in our water filters. These are filters in the closed circuit lines between our microscopes and water recirculating units. The lines are mainly in the ceiling or totally insulated as they run down the walls in the scope rooms. Imagine our surprise when we went to do a minor repair on one and watched as the plumber removed a regulator and inserted a piece of galvanized pipe. Apparently when the building was built (20 years ago), the contractor used galvanized pipe when copper had been specified. As it was hidden, we did not know about the switch. All visible lines hooking up the chiller compressor cooling with building water were copper. Well now these galvanized lines are really breaking down and clogging the water pumps. We intend to replace all but were questioning whether it would be best to replace with copper or PVC piping. Any suggestions? One concern was whether there would be the need to acid clean these lines in the future; this is done routinely to the compressor lines to remove mineral build-up. Since it is a closed circuit, we should not accumulate large amounts of minerals even though tap or deionized water will be used for the system. We also can control algae growth with chemicals. Any suggestions on this and should this dictate which material is used for the pipes? Debby Sherman <dsherman@purdue.edu> 17 Feb 2006

For our new IMAGE building, I specified PVC piping for the closed circuit loop between the water chillers and scopes. We still notice a greenish

sludge building up in the water filters. It takes about 6-8 months to become significant. I am certain that this is coming from the EM (copper cooling coils and iron connections → electrolytic reaction). The EM service people told us that if we ever used acid to clean the lines that they would no longer warranty the microscope. The PVC lines are perfectly clean. John Bozzola <bozzola@siu.edu> 17 Feb 2006

It would be good to know what the operating temperature, pressure, flow rate and the characteristics of the water feed are. There is no material incompatibility between copper piping and PVC. They can be used in the same water circuit. If you want to know if there is a problem using copper, you can take some of the water out of the system and see how much copper is present. You need to have a base line of copper from the water source, so you should take a water sample from the source also. There is likely not a problem, it depends upon your water chemistry. Knowing your water chemistry is fundamental to knowing what piping is preferred. You may have building code restrictions regarding PVC piping that is hidden, which may be the reason the original contractor put in galvanized piping. You will have to look at what codes apply to where you are. Regarding acid cleaning, you should know what your deposits are in your piping before deciding how to clean them. Copper will generally corrode at pH's below about 6.3 or so. There are low pH cleaners that can be used with copper, but they contain corrosion inhibitors. If you have copper based and iron based materials mixed in the same system, the iron will corrode preferentially through galvanic coupling, not the copper. In fact, the iron becomes a sacrificial anode protecting the copper from corrosion. Any time you have those two materials in the same system, you should have dielectric couplings between the two or you will actively corrode the ferrous based material. If you are having a copper corrosion problem, you should be looking elsewhere for the cause. David Jones <dljones@bestweb.net> 17 Feb 2006

Another material you may wish to consider is called PEX, which is a cross-linked polyethylene. I don't know too much about its characteristics, except that it's very smooth inside, which should retard crud accumulation and it's more opaque than white PVC. It may be worth checking out. Paul Grover <pgrover@bilbo.bio.purdue.edu> 17 Feb 2006

Beware that PEX will degrade over time in sunlight (ultraviolet). That said, I have PEX in my house instead of Cu, works well and is easy to run, however all transitions through the walls are Cu fittings so the PEX stays in the dark. Scott D. Davilla <davilla@4pi.com> 17 Feb 2006

I had the same sludge problem about 1.5 years ago in a new chiller for which the manufacturer recommended using only distilled water. That lasted about three months and the slime appeared. It was a combination of algae and small particles. I dumped the water and replaced it with new distilled water and Skasol to flush. Then, new distilled water and one half liter of Hexid A4 from Applied Thermal Control Ltd. UK as supplied by our SEM service tech. The chiller seized after about three months. A post mortem indicated that the impeller blades failed. This was most likely due to a misalignment of motor and pump. The manufacturer replaced the motor and pump assembly. The fluid was drained and replaced with distilled water and ethylene glycol (0.5G to 4.5G distilled water). The filters were changed and we have had no problems for about eight months. The liquid is now starting to become darker and there is a small build up of stuff in the chiller main filter. It is now time to change liquid and filters. The main filter is in the water tank and is specified at about 50 μm. The external toilet paper style filter is specified at 2 μm. Both get changed at the same time. The manufacturer specifically says to not use automotive antifreeze since it will deteriorate the BUNA N material in the chiller. Some antifreeze contains ethylene glycol. I'm puzzled by the successful use of distilled water and ethylene glycol. Perhaps they meant to say not

to use 100% antifreeze rather than a diluted mix. The other factor is that the SEM came with basically transparent water hoses. This is not good since the light gets into them and advances the algae. So this upcoming liquid and filter replacement will include replacing the hoses with opaque ones. Overall, there are three aspects to be concerned about: (1) chiller guts and pump, (2) hoses, and (3) SEM items that get chilled water (TMP, coils, etc.). I don't think that there is a single simple answer to this problem since SEMs are different and chillers are different. Gary Gaugler <gary@gaugler.com> 17 Feb 2006

I would not recommend using ethylene glycol as it is toxic. Propylene glycol is non-toxic. The problem with using "antifreeze" mixtures not intended for use in these kinds of systems is that they do not usually contain the proper additives. Glycol solutions that are not HVAC grade will deteriorate over time through a type of polymerization that will plug things up and render the system inoperable. The resulting deposits thus formed are very inert and to my knowledge no one has ever found a way to clean them so you basically have to replace the chiller system. HVAC grade glycols contain additives to avoid that problem plus inhibitors that stop corrosion of most commercially available materials in piping. That also includes seal materials, but I'm not sure about specifically N-buna seals. I'd have to look that up, but I would think it also compatible being such a commonly used seal material. Biofouling is quite common in closed loop systems. Using a biocide is called for in these systems to eliminate this problem. The original poster to this thread likely is in a location where they have a professional water treatment company taking care of large HVAC systems. Perhaps they should talk with the representative of that company and find out what is being done for chemical treatment of chilled water systems there. They may be able to just get some of the proper chemicals that likely exist on-site already. I would also like to point out, there is really no reason to go through the expense of using a glycol based system unless there is danger of freezing the coolant for some reason. There are numerous other water treatments that are much less expensive and work very well to keep a closed loop system running. David Jones <dljones@bestweb.net> 18 Feb 2006

EM – operating voltage

In electron microscopy, the higher the voltage the greater the penetrating ability of the electron beam; but the trade is a reduction of what? Naomi Piyaratna <nomy_nay@hotmail.com> 08 Mar 2006

A higher voltage will reduce amplitude contrast (see below) because the nuclei of the specimen atoms will scatter higher energy electrons less than lower energy electrons. It's quite common for biologists to use a 60 kv electron beam routinely to enhance contrast for instance, whereas other users may favor 80 kv or more for the brighter higher resolution image. So increasing the voltage seems to produce the same effect as using a larger objective aperture (which will also reduce contrast). Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 08 Mar 2006

Higher voltage decreases contrast. The scattering cross section decreases as electron energy increases up to about 800-1000 kV (depending on the atomic number of the material). This means that for a specific scattered fraction of incident electrons, the allowed thickness will be greater at higher energy; however, since for a given thickness the scattered fraction is smaller, the difference between what happens when the beam strikes your specimen and when it passes through a hole will be less, so there is less contrast. There is no free lunch. Bill Tivol <tivol@caltech.edu> 08 Mar 2006

I am been dismayed by the number of responses that answer the question as if it is for TEM. Maybe I am missing something, but TEM is not mentioned in the question. Neither is the voltage range, such as 80 KeV or 300 KeV. My knee-jerk response is: high penetration - high transmission less reflection - less surface sensitive. This would work equally well for SEM, STEM, TEM, AEM, etc. Jim Quinn <jquinn@www.matscieng.sunysb.edu> 09 Mar 2006

JOKES

- F(x) walks into a bar and asks for drink. The bartender declines stating "we don't cater for functions."
- A neutron walks into a bar. "I'd like a beer" he says. The bartender promptly serves up a beer. "How much will that be?" asks the neutron. "For you?" replies the bartender, "no charge"
- Scientifically, maybe body cells -*do*- replace themselves completely in seven years – but, legally, you're still married.

NETNOTES

The larger beam-sample interaction volume that results from a higher beam voltage results in the signal coming from deeper in the sample, rather than just from the surface. This gives information from deeper in the sample, but sacrifices information from the very surface. If you need to see small features on the surface of your sample, a lower accelerating voltage is better. I hope this helps. Any basic SEM text should cover this point. Mary Mager <mager@interchange.ubc.ca> 08 Mar 2006

In SEM, you will lose surface sensitivity with increasing voltage. In TEM, scattering cross sections decrease which is not good for EDX and EELS. Hongqi Deng <hud105@psu.edu> 09 Mar 2006

Greater high voltage in a TEM is one of the few things in nature that does not have a lot of serious "Cons" that outweigh or balance the "Pros." Granted that increased radiation concerns and somewhat less contrast attend increasing the voltage, but on the plus side, the increased penetration, easier specimen preparation, improved resolution, plus others pros are big advantages. Please forgive me if I point out that should you have a radiation sensitive specimen that you can always lower the voltage on a 300 keV TEM for that specimen, but you can't raise the voltage on a 100 keV machine to allow you to see through a thick specimen. Ron Anderson <randerson20@tampabay.rr.com> 09 Mar 2006

Sorry if I digress a bit, but I am new to the field of EDX. I thought that higher voltages gave a higher signal in EDX, and so a higher sensitivity. Is it not true? Stéphane <nizets2@yahoo.com> 09 Mar 2006

Not strictly true... It depends on your examination goals. Here are two extreme, but not unusual, examples: If I am looking for Pb somewhat deep below the surface (especially if the matrix contains S / Mo that can interfere with the low energy Pb lines), higher kV is indicated (20-30 kV). In this example, I need the high kV to penetrate deeply and to excite the higher energy Pb lines. The higher energy Pb lines will better escape from the sample as well. On the other hand, if I am trying to identify micron size B4C crystals residing on a surface, low kV is indicated (2-5 kV). In this case, I desire low penetration to minimize excitation of the substrate and minimize dilution the response. Woody White <nwwhite@bwxt.com>

I understand your explanation, but the intensity of the signal (Y axis) in EDX does not depend on the nature of the material (this is the X axis), but on the number of times the same signal is read. This means that the intensity of the signal read by EDX depends on the number of electrons that hit a certain point on the sample, per unit of time. And this depends on the current. Stéphane Nizets <nizets2@yahoo.com> 09Mar 2006

It sounds counter intuitive, but the intensity of the EDX signal only depends on the element itself and the probability of scattering events. We use a factor "cross section" to quantify such probability. Look at its expression in any TEM book you will see the higher the voltage, the smaller the cross section. Or I like to consider this question physically in the following way: Electrons can be considered as many single waves. The higher their voltage, the shorter their wavelength and the smaller the "size" of every one of them. Apparently the small ball can travel longer in certain specimen. Just like a car is much easier to get blocked by traffic than a motorcycle. Hongqi Deng <hud105@psu.edu> 09 Mar 2006

What you do get at higher kV is a better peak-to-background ratio (at least in a TEM). Characteristic X-rays are emitted isotropically. However, part of the background arises from bremsstrahlung which is forward scattered (i.e. down the column) - the degree of forward scattering is dependent on the velocity of the electrons. Hence higher kVs result in the forward scattering increasing. But, since the EDX background is not wholly dependent on bremsstrahlung, the actual instrumental gain is not as much as you would expect from a simple physics argument. In the case of SEM, you are probably best going to low kV, since this reduces the excitation volume, so improving the spatial resolution. However, this only really works with a FEG gun (to get enough probe current at low kV) and with WDX, since you have to work with L and M lines and need the resolution of WDX to separate the lines. Larry Stoter <larry@cymru.

freewire.co.uk> 09 Mar 2006

The problem with HV and 'sensitivity' of EDX measurements is very complex. First, the overvoltage $U = Eo/Ec$ (primary electron energy / critical shell ionization energy) should be at least more than 2 for all elements of interest. If not, every gain in kV is leading to extreme excitation enhancements of the element (and X-ray production). If $U > 3$, the X-ray excitation curve decreases very little. If the basic excitation of characteristic X-rays is sufficient: #1) From the point of view of detection limits you have to consider the peak to background ratio. The background in EPMA is bremsstrahlung. The ratio of characteristic line excitation to bremsstrahlung excitation is always gaining with primary electron energy (HV). Therefore you achieve always better detection limits with higher kV (but see #2, the count rate limitation can work against!) #2) You must take into account: Higher electron energy always excites more higher energy X-rays (both characteristic X-rays and bremsstrahlung). The count rate possibilities of an EDX are always limited. So you have to consider, that you possibly will get less count rates for the elements of interest with higher energy excitation... because your pulse processor has to process more high energy X-ray signals (less counts in a given time for the low energy X-rays you have in focus). E.g. The peaks are lower in a Au/Ag alloy between 2..4 keV (Ag-*L*/Au-*M*) with 40 kV excitation, than they would be with 15 kV. But the Au-*L* lines (near 10 keV) are 4 times higher (because 15 keV is very low for Au-*L*). Therefore, an increase of kV is bad for Ag-*L*/Au-*M* measurement, if a limited EDX count rate is given. #3 Also, the X-ray absorption in the specimen is increasing with higher kV. Low concentration element counts will be very much reduced behind the absorption jump of a primary element. But to drive against absorption: Tilt the specimen towards the EDX detector. The absorption influence is not so important with TEM X-ray measurements (thin specimen). Finally: The better detection limits ('sensitivity') goes really with higher kV. But one has to take into account limitations in EDX pulse processing and absorption issues (depends on matrix elements in the specimen). Therefore a simple specimen tilt is often much better than an increase of excitation energy to improve count rate yield of an element of interest. If the count rate from the specimen is sufficient, the use of a shorter pulse processor shaping-time increases the detection sensitivity (despite the worse energy resolution), because you can detect more counts in the same time. Only spectra simulation software will be able to answer these complex excitation and absorption influences in advance, without any data acquisition for tests. Frank Egert <eggert@mikroanalytik.de> 15 Mar 2006

Please don't make the mistake of simply correlating X-ray emission with a single parameter, like accelerating voltage, in either SEM or TEM applications. Your measured x-ray intensity, as a function of accelerating voltage, is a product of a number of factors that include the ionization cross-section, electron beam current, electron energy loss, the scattering path length, and the absorption path length. As the accelerating voltage changes all of these parameters will vary and you need to include all of them in any assessment of X-ray intensity for a given set of experimental conditions. The quantity that decreases with accelerating voltage is #Ionizations/nA/unit path length. Even though this quantity decreases with accelerating voltage for a constant probe size, it is likely that you will measure a higher x-ray signal as the accelerating voltage increases. For example, below the critical excitation energy (*Ec*) for a given shell, the x-ray emission for an element will be zero. It then increases rapidly to a broad maximum somewhere at ~ 2-4x *Ec*. Once you exceed ~ 4*Ec* there is indeed a decrease in the cross-section. However this decrease is NOT linear, nor is it inversely proportional to accelerating voltage. Instead it is inversely proportional to the relativistically corrected energy of the electrons ($1/2\ mv^2$), this means the decrease is not as great as you would expect. In addition, a number of electron sources actually yield higher beam currents at higher accelerating voltages, so even though the cross-section will be decreasing somewhat with accelerating voltage, the net effect can be an increased x-ray signal, until such time as the depth of production is so great that the x-ray are absorbed within the sample before being detected. If your bored and interested in seeing more detail on this for the TEM area, as well as some of the corresponding background and equations, go to the following URL http://tpm.amc.anl.gov/Lectures/ then download the PDF file XEDSAEMShortCourse.pdf and look at pages 30-33 & 44-65. Of course there are other deleterious effects of higher accelerating voltage, but that is a different discussion entirely. Nestor Zaluzec <zaluzec@aaem.amc.anl.gov> 09 Mar 2006

TEM - replicas

I have a student making C-Pt replicas. He shadows with Pt at 45 degree tilt with rotation. The protocol he has found then deposits C at 90 degrees to the source with rotation. He wants to know why he needs to change the angle of tilt to do the carbon. I could not really give him a good explanation. Can any of you help? Gregory W. Erdos <gwe@ufl.edu> 01 Mar 2006

When doing conventional replicas (static specimens, no rotation) the Pt evaporation is conducted at an angle to generate the shadows. The carbon is then evaporated at 90 degrees (directly above the specimen) to fill in the voids or gaps (shadows) generated during the Pt evaporation. This strengthens the replica. In your case, you are rotating the specimen on a turntable in both cases. Nonetheless, even though the Pt evaporation is being carried out with rotation, you will still have some gaps (otherwise you would not see any shadows). The carbon (since it is being evaporated directly overhead) will not land in the same areas as the Pt but will more uniformly coat the specimen and fill in the shadows (gaps). Although it fills in the gaps, its low density does not interfere with the shadows generated by the Pt. So, the reason for the different angles is to make the replica stronger by filling in any voids or gaps. John Bozzola <bozzola@siu.edu> 01 Mar 2006

We used to use Pt/C replicas to show surface relief. We would shadow a cellulose acetate surface replica with Pt/C at a small angle to the surface to highlight the surface texture, then deposit C normal to the surface to provide the support film. After the C film deposition, the C film was removed from the replica surface and mounted on a copper grid. It is still useful when you want to see the height of features on a surface. If you know the shadow angle, you can easily calculate the height. Henk Colijn <colijn.1@osu.edu> 01 Mar 2006

The previous responders are correct in that the carbon makes the support film and the heavy metal creates shadows of the surface structure. The heavy metal angle is really a variable and should not always be set at 45 degrees per some protocol. The shallower the surface structure, the lower the Pt deposition angle. A picture here would be a big help, but think of a 100 nm high step vs a 2 nm high step on an otherwise smooth substrate. From my experience 45 degrees would be OK to shadow the 100 nm step into visibility (actually a bit high). A Pt deposition angle of 10 to 15 degrees would be better for the 2 nm step—longer shadows. Whatever angle you use you can make a rough calculation of step height from the geometry of the Pt shadowing assuming the replica stays flat *vs.* assuming a potato chip shape. Aside from AFM imaging, a Pt-C replica will give better topographic resolution of extremely small steps on a flat surface where secondary electron SEM contrast is weak, IMHO, better than a super-duper SEM. Single-atom high growth steps on a growing crystal surface for example. There is another "lost art" replication method I would love to see someone perform and send me the images to display in Microscopy Today: (I no longer have access to an EM lab). Pull a carbon replica of a fairly rough surface. Do not apply a heavy metal shadow. Put the naked carbon film into a TEM at 100 keV or so. Tilt the specimen as high as your goniometer stage allows—45 to 60 degrees is best. Image with a small objective aperture in the bright-field position, allowing the unscattered main beam to pass. Find an interesting step or structure using the weak contrast available in this mode. Then slightly displace the objective aperture so that the bright part of the main beam is just outside the aperture (or tilt the beam leaving the aperture centered to obtain the same effect). In other words, you are

making a dark-field image using the "tails" of the main beam. Refocus. The result should be a sharp, high contrast, positive image that looks like and has the resolution of a high quality SEM image. Ron Anderson <randerson20@tampabay.rr.com> 02 Mar 2006

For those of you who were curious as to why we were using rotary shadowing, I would refer you to the web page of Gary Borisy http://www.borisylab.northwestern.edu/pages/protocols/electmicrosc.html#metcoat. Look at Figures 4, 5, 6, and 8 to see the result of such shadowing on cytoskeletal proteins.Gregory W. Erdos <gwe@ufl.edu> 02 Mar 2006

If the student is looking for macromolecules you actually need an angle of 8 to10 degrees for Pt and 90 degrees for Carbon. If he uses high angles like 45 he will not see anything. Those high angles were used for big stuff like bacteria and some of the larger viruses like TMV. I have several protocols that I can supply off line. Al Coritz <sampleprep@earthlink.net> 02 Mar 2006

If the specimen is very small, and/or has low contrast (unstained), and can be deposited onto a Formvar or carbon-coated grid, then the student could shadow the sample on-grid with Pt/C and omit the carbon altogether. The carbon layer is only important so that the replica doesn't distort or crack when it is floated off of the substrate. Because there is no extra carbon layer, there is a slight increase in contrast with an on-grid shadow vs. a true replica. As mentioned in previous replies, small samples usually look best when shadowed at a low angle (10-15 deg. from horizontal). Furthermore, rotary shadowing usually looks best on fibers while fixed-angle shadowing looks best on globular/particulate specimens. And finally, there are less steps involved in making an on-grid shadow vs. a replica. Andrew Bowling <abowling@mail.utexas.edu> 03 Mar 2006

Along the lines of the string about replicas, can Au or Au/Pd be used? Thanks, Bryan Bandli <bbandli@mvainc.com> 01 Mar 2006

I believe that they went with Pt/C to form small clusters for better resolution. Pure metals will nucleate in larger islands. Henk Colijn <colijn.1@osu.edu> 02 Mar 2006

TEM - carbon post-coating

In TEM I face 3 problems and I wondered if you could not solve them by the same solution, namely carbon-coating over the sections. My first problem is with semi-thick sections (for tomography): they don't stick to the grids during contrasting and I lose them! I thought that perhaps carbon-coating after the grids are deposited on the grid would help keeping them on the grid without disturbing contrasting? My second problem is with ultra-thin sections: when I do EDX analysis (at 200 keV) on 70 nm thick sections on Formvar film, they suffer much from the beam and usually I don't see anything when I pass into STEM mode because the area has been vaporized. I thought that perhaps carbon-coating the contrasted sections would help disperse the energy of the beam? My third problem deals with 50 nm sections deposited on grid without Formvar, which are very unstable under 80 keV. I have difficulties making Formvar films that stick to the grids; they tend to disappear in the contrasting solutions. I wondered if I could not deposit 50 nm thick sections on grids without Formvar and then carbon-coat them (so over the sections). I clean the grids by sonicating in acetone. Stephane Nizet <nizets2@yahoo.com> 16 Mar 2006

Carbon coating might not affect your semi-thick sections coming loose during contrasting, but a previous post suggested coating the grid with polylysine before placing the sections on it, and another suggested a brief acid dip to roughen the grid surface, so I'd try these. Carbon coating semi-thick specimens, however, will aid both thermal and electrical conductivity, so I do recommend that you coat your specimens, and if you are using a Formvar substrate, carbon coat that before placing the sections. From this answer, you can surmise that my answer to your second problem is to carbon coat both the Formvar before placing the thin sections and carbon coat the sections after. I would suggest either acid-dipping the grids and covering them with Formvar, or using a higher-mesh grid without Formvar, depending on how large an unobstructed field of view you need, and carbon coat the sections to increase stability of your 50 nm sections. In the latter case I even suggest coating both sides with carbon being careful not to dislodge the sections when you coat the underside of the grid, of course. Bill Tivol <tivol@caltech.edu> 16 Mar 2006

EDS - low Z peak pileup

Has anyone used Ag or Sn sputter coatings to avoid low alpha peak pile ups? Au, Au/Pd and Pt are not really good when trying to find P presence. These are also a problem for W...etc. Gary Gaugler <gary@gaugler.com> 01 Mar 2006

I've used sputtered Ag on one occasion with some success. The only catch was that after sputtering, the samples went immediately into the scope, and once they were removed from the scope were not analyzed again. I didn't do any experiments to prove this, but I assumed the Ag would oxidize rather quickly. The Ag didn't do as good of a job of dissipating surface charge on the sample as Au would have, but it was adequate for the work I was doing. Cheers, Bryan Bandli <bbandli@mvainc.com> 01 Mar 2006

What's wrong with carbon coating? Vladimir M. Dusevich <dusevichv@umkc.edu> 01 Mar 2006

I sometimes use chromium or nickel to coat biological samples for EDS analysis. Cr can either be evaporated from Cr chips in vacuum evaporator, or sputtered from Cr target in sputter coater. The *K*-shell x-rays don't overlap any elements of biological interest and the *L*-shell is very low at about 0.57 *K*eV. The Ni *L*-shell is at about 0.89 KeV. My experience with carbon coating is that it is not very good at eliminating charging on highly insulating biological samples (critical point dried, freeze-dried) and also not being a "metal" is not a good source of secondary electrons for imaging. Both Ni and Cr are very effective at eliminating charging and make for stable secondary electron images for capturing good images of what you are doing EDS analysis on. Having said that, I shall attempt carbon coating today on a biological sample to compare with EDS done on Cr coated sample a few weeks ago, and see if detectibility of trace amounts of Cu and Zn is improved. Gilbert (Gib) Ahlstrand <ahlst007@umn.edu> 01 Mar 2006