# NETNOTES

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**Selected postings from the MSA Microscopy Listserver (listserver@msa.microscopy.com) from 2/10/04 to 4/1/04. Postings may have been edited to conserve space or for clarity.**

### LM – section wrinkling

*During the past month, I have been working on semi-thick sections. To spread the wrinkles, I used Chloroform and it worked fine. I am wondering if any of you have better and safer methods to spread the thick sections. Long Miao <lmiao@bio.fsu.edu> 19 Feb 2004*

I bought a 120 V heat pen. It was expensive (~$450) but it was more effective than the small battery operated units (~$40). We use it on both thin and semi-thin sections. It is a lot better and safer than chloroform. Tom Phillips <phillipst@missouri.edu> 25 Feb 2004

Heat pens are good but you might be able to remove wrinkles by using a small wire loop to transfer the thick sections to a drop of filtered de-ionized water on a slide, then leaving the slide on a hot-plate set at about 60°C until the water is gone. Either method is much safer than chloroform. Lesley Weston <lesley@vancouverbc.net> 25 Feb 2004

### LM – uneven fluorescence illumination

*I have a question about light sources in microscopy. For fluorescence microscopy you can use mercury or xenon arcs. I am curious about the level of even illumination you can achieve with these kinds of light sources. If they were point sources, the "radial" intensity would diminish with 1/radius3, but this doesn't seem to be the case. What is the residual uneven illumination below which it is not possible to reach with traditional xenon or mercury arc style illumination? Long ago I once read that there are fluorescent samples that you could use to make a background profile for fluorescence microscopy? Are these the pieces of plastic in which a fluorochrome is embedded? Has anyone ever measured the contribution of multiwell plate bottoms to the illumination profile, due to the non-flat bottoms acting as a lens? Peter Van Osta <pvosta@maia-scientific.com> 18 Mar 2004*

Getting truly even illumination out of a conventional fluorescence arc lamp isn't a trivial task. The illumination source doesn't really approximate a point source very well. In a conventional lamp housing, one can adjust the position of the light bulb and mirror so there is an image of the arc and a reflected image of the arc coming from behind, this is defocused to provide quasi even illumination with the intensity highest in the center of the field of view. Depending on how well the objective and filters are centered, the peak intensity can wander. The most even illumination I've seen from an arc lamp is achieved by fiber-coupling the lamp output to a fiber optic bent around a large radius to homogenize the light. The fiber output provides a better approximation of a point source and the output at the end of the fiber is even. It isn't an easy task to couple an arc lamp to a fiber optic. There are fluorescent plastic slides. On an inverted microscope, one can use a dilution series of fluorochromes to test things; I prefer this approach. I use chambered coverslips to hold the fresh solutions of fluorochrome. I haven't heard of anyone measuring the optical aberrations caused by multiwell plates, but it would seem that image arithmetic could be used to quantify the difference in fluorescence intensity across the field of view using such plates as opposed to using chambered coverslips with a flat 0.17 mm borosilicate glass bottom. The fluorescent dilutions would be helpful in this context. Karl Garsha <garsha@itg.uiuc.edu> 19 Mar 2004

### EM - immunocytochemistry

*I am doing immunolocalization and in situ hybridization for TEM with gold markers. I stain with uranyl acetate. Is it possible for the uranyl acetate solution (pH 4.5) to remove some of the antibodies or probes? If so what can be done to prevent this? Alida Koorts <akoorts@medic.up.ac.za> 29 Mar 2004*

A low pH may indeed have an interfering effect on antigen-antibody interaction. You can prevent this by including a glutaraldehyde post-fixation step (2% glutaraldehyde in PBS for 15 minutes) in your protocol prior to water washes and contrasting with uranyl acetate. Peter van de Plas <p.vandeplas@aurion.nl> 29 Mar 2004

In all my immuno-EM labeling (usually on cryo-sections but also sometimes on plastic sections), I always fix the sections in 1% glutaraldehyde (in PBS - 5 min) after the final washes that follow the protein A-gold labeling step. I then rinse the sections extensively with water (because phosphate will make UA precipitate), usually 5 times 2-3 minutes each, then stain my sections with uranyl acetate and air-dry them or embed them in methyl cellulose/uranyl acetate. To answer your question, I think antibody and gold complexes can withstand a few quick washes in water and incubation in uranyl acetate, but I am sure some of it is falling off. With the fixation step that I describe above, you maximize labeling efficiency and avoid taking chances. It's always better being safe! Marc Pypaert <marc.pypaert@yale.edu> 29 Mar 2004

A few years ago, the acidic uranyl acetate step as a possible source for reduced immunodetection was subject of a vibrant and cheerful discussion on this list. I still have fond memories of that one.... To keep it brief: acidic conditions may uncouple the antibody/antigen complex, and this is more likely to happen when the acidic environment is chaotropic. Such conditions are put to good use in eluting bound antibodies from affinity columns for antibody purification. If this should happen on specimens it should be easily prevented using a glutaraldehyde step after the wash steps following the secondary gold reagent. In this way antibodies and gold reagents will become covalently linked to the specimen. Jan Leunissen <leunissen@aurion.nl> 30 Mar 2004

I agree with everyone that low pH should in theory have the effect of stripping antibody or protein A from sections. So in theory, it would be wise to fix the labeled sections with a cross-linking agent, such as glutaraldehyde, before final contrasting in low pH uranyl acetate. However, the situation remains the same as when we first discussed this issue. There are no published data to support the theory. We left the discussion last time with your comment that adding the final 10 min fixation step does no harm, so just do it. I agree with this. This still leaves the issue unresolved. I can label cryosections or Lowicryl sections with antibodies and protein A gold but cannot differentiate (subjectively) from the levels of labeling on sections treated with glutaraldehyde with those that were not. This means that treating the sections with glutaraldehyde may not have such a drastic effect on labeling as theory suggests. The field is open to anyone who wants to compare labeling efficiency on aldehyde-fixed, labeled sections with unfixed sections. It seems to be an easy experiment to do and is worthy of publication. Paul Webster <pwebster@hei.org> 29 Mar 2004

### TEM – visualizing DNA

*I am currently looking at liquid polymers in which DNA has been incorporated. In order to look at the resulting structures, I have tried several negative stains such as PTA, uranyl acetate, ammonium*

*molybdate with different results and none of these really satisfy me. For example, I have difficulties getting the sample to actually stay on the Formvar grid; most of it seems to slip away. The method I use is the following: sample sitting on the Formvar grid for 1-2 minutes; drain the excess with a filter paper apply the stain for 1 minute; drain the excess with a filter paper. I do not have access to a high vacuum evaporator (shadowing technique) but someone told me about using a colloidal carbon mix with the liquid polymer sample, similar to negative staining. Has anyone heard about that technique? Diane Montpetit <montpetitd@agr.gc.ca> 19 Feb 2004*

I know nothing about "colloidal carbon" - does it exist in this world? On the other hand, I doubt that even "colloidal carbon" could help you. I suppose you want to see the fine details of your sample if were trying to use negative staining. DNA is 2.4-3 nm in diameter. Even naked DNA may not be visualized well using negative staining. You may see DNA using "positive staining" but it's quite difficult and you need to use high-resolution STEM and probably Z-contrast. You would never want to use Formvar support film! To see any DNA on a polymer's background is a big problem because your polymer will scatter most electrons. In general, you may not use any plastic support film for high resolution TEM. You need to use thin carbon support films for precise work. I would suggest that you need to try freeze-fracture in combination with good SEM. It will give you an idea of the 3D structure of the polymer and you probably will be able to see some DNA conglomerates (not DNA strands). Sometimes DNA creates a sort of periodic structure which you might also be able to see. I know investigators who have frozen polymers, prepared ultrathin sections and analyzed them by TEM after staining with OsO4 or UA. Sergey Ryazantsev <sryazant@ucla.edu> 19 Feb 2004

DNA won't stick to Formvar. One must use Collodion, which is some form of nitrocellulose. It is also barely possible to visualize it by staining. The 1.5 nm diameter limits this technique, not because it is below the resolving power of the EM, but because one cannot get enough stain on the double helix to distinguish it from background by conventional imaging techniques. Carol Heckman <heckman@bgnet.bgsu.edu> 20 Feb 2004

**TEM – uranyl acetate solubility**

*I continue to have problems getting uranyl acetate into solution (aqueous or acetone) so that it not only goes in but stays in for a reasonable period of time (weeks preferably). Concentrations can vary from 0.5% to 2% but the problem remains. I have tried uranyl acetate from a number of different sources and still have minimal luck. What we do now is put the required amount into the solvent and then let stir on a magnetic stirrer, often for hours. Then we filter out what does not dissolve. Of course this leaves an unknown concentration in the final solution. Keeping the solution dark does help slow down precipitation but it still occurs and this does not help with the initial dissolving of the reagent. One solution seems to be to add acetic acid to the water to lower pH. The pH of water, although usually acidic, does vary depending on the purification method. Does anyone know the optimum pH for dissolving UA? This solution however will not work with acetone when you want to add UA for freeze substitution. Perhaps we need to forget the percentages listed in all the methods and just admit that we are using "saturated" solutions of UA or report the pH just like you do with other solutions. Does anyone have a brand to recommend that dissolves*

*well or any special tricks? Debby Sherman <dsherman@purdue.edu> 23 Feb 2004*

Uranyl acetate is quite soluble in water. 1-2% (w/v) aqueous solutions are stable in the dark at +4°C for a few months. Usually I dissolve uranyl acetate in a plastic tube with gentle shaking. It's completely dissolved in about 40 min at room temperature. I think it is a water problem. I usually use 20 Mohm/cm2 "cell culture" grade water. Usually, at the normal circumstances, water exposed to air is pH 5-5.5. Basic water pH usually indicates bacterial contamination (on the filter, lines etc). In Russia we used to use double-distilled in quartz water without any problems. According Merck Index, uranyl acetate is soluble in 10 parts of water, which means 10%. Sergey Ryazantsev <sryazant@ucla.edu> 23 Feb 2004

We have always used a saturated solution of uranyl acetate in water for staining grids; when we make it up, we stir overnight, then once it settles, we use the "clear" stain. For use, we dilute the saturated uranyl acetate 1:1 with 100% methanol and then filter through a 0.4 micron syringe filter into the Hiroaka staining trough. We have never had bad results. Margaret Sherwood <msherwood@partners.org> 23 Feb 2004

In the uranyl acetate that is commercially available, there is always a small amount of totally insoluble (in water) "contaminant" - that according to my vendor whom I discussed this with about 5 years ago. So when I mix up 3% uranyl acetate in distilled water, I stir it on a magnetic mixer for an hour, add 1 drop of concentrated glacial acetic acid per 10.0 ml of stain to reduce long term uranyl precipitate formation. I then let it stand overnight and carefully pipet off the clear uranyl acetate into a clean, clear glass bottle that I store inside a dark box. It will stay clear with no precipitate gathering on the bottom of the bottle for about 1-2 months, then maybe a real fine layer may be discerned on the bottle bottom, at which point we filter it through 0.2 micron filters as we use it. By the way, I collected some of that insoluble component that settled out during the night after dissolving the uranyl acetate, washed those crystals with distilled water to get off any residual uranyl acetate, and did EDS on them in my SEM/EDS machine. All crystals examined had high to medium amounts of titanium, silicon and uranium in them, medium amounts of oxygen, low amounts of iron and aluminum, some with low phosphorous. So the crystals are probably a mix of 2-3 types of an insoluble uranium compound. As for ending up with unknown concentration from filtering, you're probably still pretty close to the 2% target you use, and if you mix up the same way and amount each time and stain for some empirically determined time, at least you'll be consistent. In sum, there will always be some insoluble crystals left when dissolving uranyl acetate so handle as above to minimize or eliminate precipitates from that source on sections. Gib Ahlstrand <ahlst007@tc.umn.edu> 23 Feb 2004

**TEM - KeV vs. chromatic aberration**

*Could someone explain to me how using a higher accelerating voltage decreases chromatic aberrations in the EM? Unless we're talking really low KeV (i.e. 100ev - 1,000 eV vs. 100,000 eV - which is why I suspect one reason why low eV in SEM's is generated by decelerating the electrons at the bottom on the lens system) why would the energy spread of the primary electron beam vary? Richard Edelmann <edelmare@muohio.edu> 02 Mar 2004*

We just covered this a few weeks ago in my course: The equation for the diameter of the disc of least confusion (d) for chromatic aberration is: d = Cc . alpha x (delta E / Eo) where Cc is the coefficient for chromatic aberration, alpha is the convergence angle of the beam, delta E is the energy difference, and Eo is essentially the beam energy. For thermionic emission from a tungsten filament, the initial energy of the electrons varies between about 0 and 2 eV or so; this is, I believe, a function of variations in their initial thermal energies within the filament. As a result, if the accelerating voltage is 2 kV, (delta E / Eo) is 0.001; if the accelerating voltage is 20 kV, (delta E / Eo) becomes 0.0001; and if the accelerating voltage is 200 kV, (delta E / Eo) is 0.00001. Thus, the diameter of the disc of least confusion for chromatic aberration is basically inversely proportional to the accelerating voltage you're using. From: Ellery Frahm <frah0010@umn.edu> 02 Mar 2004

I'm not sure if this is what you mean. But if the resolution (chromatic) = constant x focal length x semi angle x delta V/V then if a finite change in voltage occurs (delta V) increasing V must improve resolution. Part of this may due to the optics of the system but a major consideration is the thickness of the specimen where I'd always understood that the loss of voltage was roughly finite (I won't say linear) then increasing the overall accelerating voltage should improve resolution. This does work even at 60kv+. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 02 Mar 2004

**TEM – polymers**

*We have to confirm the identity of polypropylene and polyethylene in a film embedded in a resin using TEM. We cut with the ultramicrotome the sections and stain with OsO4 for 30 minutes. The results are not good because there is no reaction with the OsO4. Belén López Mosquera <sxaimic@udc.es> 03 Mar 2004*

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I would try staining with Ruthenium Tetraoxide (RuO4). Even though both polymers will stain they should stain at different rates. According to "Ruthenium Tetraoxide Staining of Polymers for Electron Microscopy" by Trent et al. 1983 Macromolecules16: 589-598, polyethylene oxide will stain faster than isotactic polypropylene. I would stain only for between 5 min and 15 min. Both RuO4 and OsO4 are very dangerous chemicals so please be sure to observe proper safety procedures. Also you will need to use a cryo-ultramicrotome or the polyethylene and polypropylene phase will smear. Stephen McCartney <stmccart@vt.edu> 03 Mar 2004 One way of looking at polymers, especially hydrocarbon polymers like polypropylene and polyethylene, is to prepare a surface and etch with a permanganic reagent. The etched surface is then either replicated and the replica examined under TEM or gold coated and examined under SEM. It is very easy to distinguish polyethylene and polypropylene this way. We have a picture gallery of etched surfaces, albeit for specimens with special thermal or mechanical treatment, on: http://www.personal.rdg.ac.uk/~spsolley/Picture_Gallery/new_pgal.html Robert H. Olley <r.h.olley@reading.ac.uk> 4 Mar 2004

**TEM - resin infiltration**

*Does anyone have any trick/tips that they would share about promoting resin infiltration? I work in a clinical pathology lab and time is of the essence. I had read a small blurb in a book about someone putting their straight resin and sections during infiltration under a 100 watt light bulb for the heat to make the resin less viscous. Is this an OK thing to do? What do most people feel is the most important step in resin infiltration---the straight resin step vs. the step with the resin mixed with propylene oxide? Which step should I be placing the most emphasis on to keep my time in the infiltration step as short as possible and yet get the best possible result? I use EMBed 812 epoxy resin, propylene oxide, and process soft tissue for clinical diagnosis--tumors, kidney, muscle, nerve, etc.) Karen Bovard <kbovard@creighton.edu> 10 Mar 2004*

When I worked in a Clinical EM Lab we found that placing our specimens, in 100% resin, into a vacuum oven set at 37 degrees C for an hour helped tremendously. The heat and vacuum together work much better than leaving the vial on the rotator. Also, for large nerve pieces, you could leave them on the rotator overnight in the 1:2 propylene oxide:resin then place them under vacuum the next morning. That helped to prevent many of the holes in the axons. As to which step is most important, the propylene oxide and resin or 100% resin, they are equally important. The propylene oxide removes the alcohol or acetone, as well as making the resin less viscous to pull it into the tissue. But the 100% resin, especially under vacuum, draws out the remaining propylene oxide and further infiltrates the tissues. If you have any propylene oxide left behind your blocks will not polymerize properly nor cut well. Donna R. Clarkson <donna.clarkson@brooks.af.mil> 10 Mar 2004

I think the fastest and easiest way to promote resin infiltration is to use a microwave system, although it does require additional

equipment. Microwaves can take you from fresh tissue to polymerized blocks in 4-6 hours, with equal or sometimes better ultrastructure. They are ideal for diagnostic work, because they make the procedure very fast, compared to conventional processing methods, and do not (in my experience) compromise quality. You can find details and protocols for these systems on the websites of vendors selling them. Randy Tindall <tindallr@missouri.edu> 10 Mar 2004

The heat of the light bulb should not be a problem because I routinely put my embedding dishes on top of my 60 degree oven overnight to warm and thin the Epon before putting the dishes into the oven. I remember when I started in TEM that our procedure called for putting the molds into 2 ovens, the first was maybe 35 degrees and the second at 60C the next morning. Decades ago, a fellow technician was doing a fast sample prep of melanoma tumors. He needed to section the day after receiving the tissue so he trimmed the samples as small as possible and then used a magnetic stirrer in a scintillation vial to keep the solutions moving all the time. This worked up to the complete Epon exchange which was really too dense for the stir bar to move in. Of course all the times were shortened so that the samples went into the oven by the end of the day. If I remember correctly the oven that he used was set at 70°C. For years I have put difficult samples on a rotating table instead of the table top since I do not have one of those tissue rotators. The motion helps to mix the small amount of solution that remains in the vials into the newly added chemicals. Can you purchase a microwave oven? Pat Connelly <psconnel@sas.upenn.edu> 10 Mar 2004

**SEM – soil samples**

*How can we best prepare our soil samples for SEM and micro analysis? We are novices at this. We are interested in phosphorus and iron in river sediments. We are having difficulty in interpreting the results we have been getting. We crushed soil to double sticky tape, mounted on Al stubs and coated with AuPd. The preps look good at the SEM with little charging. What we see is grains and some organic matter (OM) and coatings. The spectrum shows some OM and SiO2 along with other particles of mixed composition. My question is how can we interpret this? Carol Bronick <cbronick@vsu.edu> 3 Mar 2004*

First of all, Au lines will overlap with P, so in this case coating with Au is not acceptable. I am not sure about a goal of your research. If you need just to determine whether Fe and P are in your samples, then EDS is not the best choice of method. Depending on anticipated levels of elements the XRF, wet chemistry or even mass spectrometry could be a better choice. If detection limit of 0.5% is OK, then I would use high intensity beam current (so that dead time is about 20-50%) to acquire spectrum from pretty big field of view, at magnification of x100 or x200, for at least 5 min. Sometimes fast mapping (again at high beam current and at magnifications when many particles are visible) can help to localize the place of interest. Vladimir Dusevich <dusevichv@umkc.edu> 3 Mar 2004

Actually, P *K*-alpha and Au *M*-alpha don't overlap but are indeed very close by 108eV. Pt might be an option but its M alpha is even closer to the P K alpha. The 108eV distance at low eV is not always a problem depending on your system. Some systems come with powerful tools for performing peak pile up deconvolution. A quantitative EDS analysis with low intensity errors will give very good results. Any element that has high (>20%) intensity error should be discarded or re-run at different KV. At F, my detector is about 56-59eV (give or take) resolution and how one calculates this. Calibrated resolution at Al and Cu is typically 128eV at 102uS. Potting the soil in metallurgical mounting media then polishing should provide good results. The irregularity of un-polished (3-D) soil grains is not good for EDS. Flat, polished surfaces are best. Collect at 102 μS with at least 1,000 cps and do it for about 300 live seconds. Keep DT < 25%. Then do peak ID, peak deconvolution and finally, quantitative EDS analysis and look at the Intensity Error values. I look at C, F, O, P, Al, Si, Fe, S, Cu, W, Ta, Hf, Zr, and many others (not all at the same time!) that are Au/Pd coated. At 2X KV of highest value, I can't recall a time that I could not define the constituents with my EDS. Organics/polymers are another story; you need FTIR or WDS for that. Gary Gaugler <gary@gaugler.com> 03 Mar 2004

We have done quite a bit of this so far. Most investigations were done on sediments ranging between 1 m below the surface up to rocks at ~100 m below the surface. My preference is still to mount in Araldite (the cheap version you can get from fiberglass hobby shops) under vacuum to get rid of trapped gas and polish to a 1 micron surface finish. Do not crush. You lose information like pore density and distribution/relationship of the different components. Weathering is clearer in a cross section. Most of the investigation is in BSE mode. Carbon coating is preferred since it interfere less with the EDS spectrum. If you can work in low vacuum range (0.1 torr - 1 torr) it helps with reducing charging.

A reasonable reference is (more suitable to rocks) is "Backscattered Scanning Electron Microscopy and Image analysis of Sediments and Sedimentary Rocks" by D. H. Kringsly and other authors. Cambridge Press ISBN 0-521-45346-1. S H Coetzee <coetzees@mopipi.ub.bw> 4 Mar 2004

**EM – STEM vs. FE-SEM resolution**

*Does STEM (scanning transmission electron microscopy) give better resolution than the best FE-SEMs? David Patton <David.Patton@uwe.ac.uk> 30 Mar 2004*

Yes. With a thin TEM specimen the excitation volume is negligible for a STEM image. The present 'Industry Standard' for high resolution HAADF (high angle annular dark field) STEM is Si dumbbells at 0.136nm. With Cs correction, probes of around 0.1nm can be obtained in these instruments. Of course, HAADF imaging is not the same as secondary imaging used on an SEM and the secondary signal from a thin specimen would be low but in terms of resolution STEM instruments are better. There are (at least) two sites in UK (SuperStem at Daresbury and Dept. Materials at Oxford) as well as others around the world who can achieve this performance. Ron Doole <ron.doole@materials.oxford.ac.uk> 30 Mar 2004

Indeed! As Ron points out, high-end HAADF STEMs can achieve resolutions at, or even below, 0.1nm. Larry Allard and I had the honor and task of organizing the session on Advances in High Resolution Imaging at last year's Microscopy & Microanalysis meeting. We had several talks from STEM folks that showed such resolutions -- check the High Resolution Imaging abstracts in last year's M&M proceedings and also the paper by Phil Batson in Nature: Batson, P. E., Dellby, N. & Krivanek, O. L., Nature 418, 617-620 (2002). This year's M&M meeting (in Savannah, Georgia) will also include a session on High Resolution Imaging (both STEM and TEM) organized by Larry Allard and Jim Bentley. In addition, there will also be a pre-meeting congress on Cs-corrected electron microscopy. For information, click on "Next TEAM related discussions July31-August 1 2004 in Savannah" at http://ncem.lbl.gov/. Mike O'Keefe <maok@lbl.gov> 30 Mar 2004

A SEM-based STEM at 30kV can achieve subnanometer resolution (~0.8nm). This is typically a solid state detector that integrates with an ultra-high resolution SEM. A dedicated STEM or TEM/STEM (200-300kV) can achieve ~0.8A resolution. Edward Principe <eprincipe01@hotmail.com> 30 Mar 2004

**SEM - charging phenomenon**

*Does anyone have information about the charging phenomenon of SEM? I know it's because of the secondary electrons yield, producing a dark rectangle on image, when setting a too low voltage (low electron energy), and a bright one, appearing like a false relief, at a too high voltage (high electron energy). But, is that all? Sylvain Maury <sylvain.maury@thalesgroup.com> 08 Apr 2004*

I have just spotted your question and hope I am able to provide a simple explanation? It is not correct to say that an accelerating voltage is too low when viewing an image at a single kV. The only time that this comment may be justified is when, having increased the accelerating voltage, you see the subsurface detail that you desired. In another case when considering EDS, the accelerating voltage may be too low to stimulate the specific peak that you may desire. I do not believe there is such a situation that a kV is too low, but of course it may be too low to display the information that you require. In general most operators of non FEG instruments run at too high a kV to truly resolve the specimen surface (i.e. >5kV)! There are other interesting reasons for contrast changes within images at very low voltages, these relate to secondary electron emission coefficients and are well presented by N.R. Whetton in Methods of Experimental Physics, Vol. IV (1962). The dark patch on the image at low accelerating voltages is due to contamination that has deposited on the specimen surface. The contamination is invariably a lower emitter of electrons, thus it shows up as a dark patch or line. You do not see this at a higher kV because the additional voltage causes the beam to penetrate further into the specimen, the sub surface information generated dominating the image hiding the "surface" contamination from your view. To see contamination is an indication that you are seeing the "true surface" of the specimen. Charge on the specimen surface is due to an insufficient earth leakage path in relation to the incident beam current and you are correct in the belief that this may give rise to a bright square on the image. Another visualization of charge is the bright particle with a black halo around it. Here the charge field that has built up around the particle is preventing the low energy SE escaping, whilst if you look closely the high energy BSE do escape and provide information within the charge (black) cloud. In a charge-discharge situation, often seen on slow scans, the image will dim (through a reduction in SE emitted) as the specimen charges, flashing bright when the system discharges due to the sudden freeing of the SE held under the charge. Thus a progressively darkening area in an image indicates charge; the bright flash across one or more lines indicates discharge! Steve Chapman <protrain@emcourses.com> 10 Apr 2004