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Selected postings from the MSA Microscopy Listserv (listserv@msa.microscopy.com) from 06/15/06 to 08/15/07. Postings may have been edited to conserve space or for clarity.

SPECIMEN PREPARATION - colloidal gold conjugation of proteins

I would like to prepare a protein-gold (5 nm) complex and to follow internalization of the protein into the cell. I used the protocols described by Hayat (Colloidal Gold, Principles, Methods and Applications) and previous recommendations of M. Bendayan, but I failed... The protein is a small protein of 10 kDa with a pI of 8.5 and is dissolved in water. In parallel, I performed a control BSA-colloidal gold complex and it is OK. I tried to work with a pH of the gold solution slightly basic to the isoelectric pH of the protein, pH=9. I also tried to work at a pH of 7.4 and I failed. In both cases, I added the colloidal gold (pH=9 or pH=7.4) to the protein solution. As recommended by M. Bendayan, I did not use any PEG or other protective agents. I then centrifuged the mixture at 45,000 rpm at 4°C for 60 min. The sediment at the bottom is dark-red. I recovered the sediment in PBS containing 0.02% PEG 20000. When I checked with negative staining in the TEM, I did not visualize any gold particles or very few! Is it the molecular weight of the protein that is the problem? Is the pH of the gold solution not optimal? Is a protective agent required to stabilize the mixture before centrifugation? Jeannine Lherminier <lhermini@epoisses.inra.fr> 19 Jun 2007

I don't think the problem lies with the MW of the protein or the pH of 9 for conjugation. To better address the question, I need a little more info. First, was a concentration isotherm performed at either pH to determine the amount of protein necessary to stabilize a given volume of cAu against electrolytic flocculation (i.e., influx of saturated NaCl)? This by itself will go a long way in telling whether stable conjugates are present. If the conjugates remain red in color above a certain protein concentration following addition of salt, then it would seem that the reason very few particles are visualized by TEM is that either the sedimented conjugates were too dilute following resuspension, or they were not allowed to adhere to the grids for a long enough period of time. For labeling experiments, we generally resuspend to one-tenth of the original volume of cAu, giving a particle concentration of roughly 1013 per ml. We have found this minimizes the amount of time required to saturate all binding sites on a cell surface. At this concentration, a grid should only need to sit on a drop of the conjugate for 20 minutes or so at room temperature for there to be plenty of particles for imaging. To visualize the protein coat around the particles by negative staining, the PEG has to be omitted entirely in order to be sure that whatever surrounds the particles is definitely protein and not PEG. If the conjugates are not stable in PBS without PEG, then resuspend in water to do the negative staining. If PEG is needed as a secondary stabilizer in PBS for the labeling, I add it after conjugation, but before centrifugation. However, remember that it is important to do the initial concentration isotherm in order to be sure that protein is bound to the particles, and that it is not simply the PEG that's stabilizing them. Daryl Meyer <dameyer@wisc.edu> 20 Jun 2007

SPECIMEN PREPARATION - paraffin dewaxing

I work mostly with plastic resin embedded tissue and only sporadically with paraffin. Is there a rule of thumb for how many slides can be dewaxed per volume before it is necessary to switch solutions? I understand the concept of moving xylene bath 3 to bath 2 and bath 2 to bath 1 and fresh into bath 3 but at what point? Tom Phillips <phillipst@missouri.edu> 13 Jul 2007

It depends on the thickness of sections and number of sections per slide. We tend to change after about 20 - 25 slides, of 6 µm, with sections covering 75% of an area approx. 20 × 45 mm. using xylene as the solvent. I bet it changes if you use an oil based solvent. Russell Spear <rzs@plantpath.wisc.edu> 13 Jul 2007

On the automatic Leica stainers used, and in the manual procedures set up in our standard operating procedures, the magic number was 400 slides, volume of each station approx 450 ml, 3× xylene, 2× 100% ethanol, 2× 95% ethanol. Roger Moretz <rcmoretz@gmail.com> 13 Jul 2007

In our lab, we change the solutions every 300, maximum 400 slides (500 ml) or once per week, depending on which limit is reached first. Sven Terclavers <sven.terclavers@med.kuleuven.be> 13 Jul 2007

SPECIMEN PREPARATION - measuring resin components

How do you all measure out embedding resin catalyst? I have only been doing it for 31 years... I used to use tuberculin syringes—had to hurry because they sort of melt. The current crop of facility users seem dumbfounded that I would use anything but a pipetter. But I have now had another new pipetter get gummed up with some resin component, probably catalyst. Does anyone have a favorite method? Tina (Weatherby) Carvalho <tina@pbr.hawaii.edu> 17 Jul 2007

I remember those days of plugged pipettors! To eliminate this we weigh all resin components into a disposable beaker. We can get close enough with a decent 3 decimal place scale to get consistent results and use only disposable pipettes for all components. Our scale sits in a hood and is protected by a draft shield. This has gotten rather messy over the years as students occasionally use acetone on the polyethylene shield to clean up spills but it still works. A piece of aluminum foil on the pan has worked to prevent resin from getting onto scale components that would affect function. Debby Sherman <dsherman@purdue.edu> 17 Jul 2007

Glass transfer pipettes and rubber bulbs. Measure by weight. Phil Oshel <oshel1pe@cmich.edu> 17 Jul 2007

I use a pipettor, but I use the big ones (1000 microliters) to measure out 300-600 microliters of catalyst so that I don't risk pulling the gooey stuff up into the works. If I need to make a large volume, I measure out the catalyst in 2 shots. Simple, but fairly effective. I haven't had a gummed-up pipettor in a while. Leona Cohen-Gould <lcgould@med.cornell.edu> 17 Jul 2007

Trying to weigh the catalyst in the fume hood, I have a problem of air flow of the hood disturbing the balance reading. I once held my nose (away from the hood) and measured the number of drops of catalyst from a particular smallish PE disposable pipette required to make the desired weight. It is very repeatable if you let the drops form (don't squirt) and use the same style pipette. I often mix resin batches in the 50cc range that require 0.2g of catalyst. I weigh the main components out in the lab (scissor off the tip of larger disposable plastic pipette for the viscous resins), mix well, then move to the hood and add the drops of catalyst. For my pipettes and DMP-30 or DMAE I use 12 drops for 0.2g. Since Spurr's resin works fine with half the normal catalyst for "Long Pot Life" mix, the tiny variations in the drop method certainly will have very little effect on the final product. Dale Callahan <dac@research.umass.edu> 17 Jul 2007

It seems that in many ways my lab may be unique in its way of handling resin. Many years ago I threw out the concept of solutions A and B, and the WPE values and went instead to a method first proposed to me by Sus Ito at Harvard. The method has worked consistently on three different continents and on a few EM course in different parts of the world. It consists of mixing three resin ingredients (but not the catalyst) in a large volume, aliquoting the mixture into small glass, screw-top vials and storing them, tightly closed, at -20°C for as long as it takes to finish the batch. As resin is needed, a vial containing from 4 to 6 ml of resin is removed from the freezer and warmed in the oven. The actual amount of resin in the vial can be determined by comparing the resin level with a calibrated and marked black vial). It is important to know the amount of resin in the vial because this will determine the amount of catalyst to use. Difficult to embed specimens can be soaked in the resin without catalyst for many days, or the resin can be mixed with catalyst and used immediately. The advantages are: 1. no messy resin preparation needed for each experiment. 2. no wasted resin components. 3. less handling of toxic components. 4. idiot-proof system ensuring reproducibility. 5. consistent results - the first vial is the same as the last vial. 6. no difficult measuring of catalyst (or any of the other components). 7. cleaner than other methods. Want to know the



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method? Here it is: Epon resin (simplified recipe for making large batches of premixed resin). Mix the following in a large glass jar (see below): NMA 110 mL DDSA 130 mL Eponate 12 230 mL. When the ingredients have been well mixed, the resin is poured into small tubes in aliquots of 4 to 6 mL and stored frozen. For use, warm a tube and, using a glass Pasteur pipette, add four drops of BDMA per milliliter (or 2 drops of DMP-30 if preferred). Mix well, use immediately and discard unused resin (polymerization is a good idea). Preparing and storing resin this way will ensure that resin ingredients are not wasted and all the tubes will have resin of the same consistency. The mixture can be tested before use by polymerizing one tube of resin. All subsequent tubes of resin will have the same polymerization and sectioning qualities as this first tube. Glass jar tip: A simple tip for measuring the resin components is to pre-calibrate a glass jar. In a clean jar, pour in 110 mL of water, mark the meniscus. Add an extra 13 mL of water and mark the new meniscus, and finally, add 230 mL of water and mark the meniscus. (For those in the USA, Smuckers jars work well - UK, Robinson's orange marmalade - it just has to be clear glass!) Pour out all the water and dry the jar well. It is now ready to be used for measuring out the resin components and mixing them. Mix the three components well before pouring the resin into the small vials. As the volume is estimated just prior to the resin being used, there is no need to get into complicated pipetting methods, just pour carefully. As for glass pipettes (and I don't mean to be confrontational with the following comments), I have been using them for many years in my specimen preparation protocols. In addition to distributing catalyst, they are very useful for collecting scraped cells from culture dishes, and for transferring solutions from around very small specimens. I am aware that glass fragments are supposed to enter embedded specimen blocks and damage diamond knives, but most people I know also trim their specimen blocks using glass knives, which is a more reasonable source of glass contamination. In all the years I have been using glass pipettes I have never come across glass fragments in embedded tissues or cell pellets. I have seen hairs, dust and large chinks of keratin, but no glass. If glass were present in the blocks, I am sure it would be obvious in the initial trimming and facing off, which, incidentally we do with an old diamond knife. Paul Webster <pwebster@hei.org> 18 Jul 2007

We also freeze large batch of resin the same way or almost. The only difference in that we aliquot the resin in syringes, which are already calibrated. The syringes are then closed with Parafilm and frozen at -20°C. Syringes are useful because you can use force and you're less likely to make mistakes with the volumes (unlike pipetting) and also there will be no remains sticking to the sides. I must say that I don't even care about warming them up in oven, I just take them out of the freezer and the resin is fluid after 30 min room temperature (just the time to concentrate on your experiment while you prepare a cup of coffee). Stephane Nizets <nizets2@yahoo.com> 18 Jul 2007

Dear Stephane, you make no mention of when you add catalyst to the resin. Do you incorporate it into the final mixture that you freeze, or do you add it just before you use the resin? There is a big difference. If you add it to the resin before freezing, the resin will slowly harden, even at -20°C. This means that the properties of the resin are changing during storage. It also means the resin mixture can't be stored for long periods. We are able to store our uncatalysed resin for more than a year. A possible complication of storing resin in plastic is that some plastics contain unincorporated plasticizer that could potentially inhibit, or otherwise alter the polymerization of the resin. Usually this is only a problem when setting up a new lab where the consumables are not exactly the same. One unique example of plasticizer interfering with polymerization is when polymerization of Lowicryl is attempted in new Eppendorf tubes. The new tubes have a volatile substance present that completely inhibits polymerization. The solution is to either autoclave the tubes, heat them in an oven overnight, or leave them in a cupboard for a few years. Paul Webster <pwebster@hei.org> 18 Jul 2007

We weigh out all our resin components and use a disposable pipette to weigh out drop wise. When working with a new resin mix for the first time if it does not have weights (only volumes) we carefully weigh out each

volumetrically measured component and write it down. Richard Edelman <edelmare@muohio.edu> 18 Jul 2007

In my earlier reply to Tina, I addressed only her immediate question: how to measure out the catalyst. In response to Paul's posting, I do a very similar thing, except that I use a method taught to me by my first boss, the late Tom Robinson: I store the resin in the freezer, in syringes. I cap the end (you can buy syringe caps from the suppliers), and wrap the tips with Parafilm. Works like a charm. If you use Spurr's resin, you can even store it with the catalyst mixed in for a few weeks at -20°C. The syringes, obviously, make measuring the volume quite easy. Leona Cohen-Gould, M.S. <lchgould@med.cornell.edu> 18 Jul 2007

My lab has been using a system similar to Dr. Webster's for 30 years. The "Epon" resin components, minus the accelerator, are made up in batches of 200-400 mL, thoroughly mixed and placed in glass vials in commonly used aliquots (5, 10, 15, 20, 30, 40 mL). Some vials are left half filled to allow for 1:1 resin mixes. They are tightly sealed and stored at -20°C. Before use the vials are allowed to come to room temperature and DMP is added. The DMP is measured in disposable 1 mm BD tuberculin syringes, without needles. At about a nickel each, why wash glass syringes? As pointed out by others, the resin can be stored much longer without the accelerator. And even before resin stored with the accelerator added becomes unusable, it will be partially polymerized and more viscous than it should be. We also use disposable glass pipettes without problems. Ralph Common <rcommon@msu.edu> 18 Jul 2007

SPECIMEN PREPARATION – fixation of low pH extremophiles

Once upon a time I had to perform TEM on pineapples of different stages of ripeness, and I had problems that someone said was because glutaraldehyde doesn't fix well at low pH. Now I have clients who want to fix bacteria that are being cultured at a pH of about 3.0. Do any of you have any suggestions for a fixation protocol? Cryo is not an option at this time. Tina Carvalho <tina@pbr.hawaii.edu>

I would still try fixation at neutral pH! Would you expect the change of pH to have dramatic effects? In parallel you could still try to fix at pH 3. Surely glutaraldehyde won't be at its maximum efficiency but if you increase the fixation time, it'll probably work. Just compare the results at both pHs. And please let us know what came out. Stephane Nizets <nizets2@yahoo.com> 19 Jun 2007

Uranyl acetate may be worth trying, since 1% water solutions have a pH around 3.8. Yet remarkable efficacy has been reported for uranyl acetate at pH 7.2 as a primary fixative for whole tissue structure and immunolabeling by Fassel & Greaser 1997 (Microsc Res Tech 37:600-601) and at unadjusted pH 3.8 as an agent for time-resolved fixation of fleeting intermediates during rapid structural transitions when used for negative staining by Zhao & Craig 2003 (J Struct Biol 141:43-52; J Mol Biol 327:145-58). Mike Reedy <mike.reedy@cellbio.duke.edu> 21 Jun 2007

Tina's query has brought to mind a parallel question that we have been pondering. Is the exact pH of the fix buffer important in fixing standard biological tissue (not extremophiles). Over the years, I have followed protocols that used pH 7.6 for some tissues (vertebrates and invertebrates), and pH 7.4 or pH 7.2 for others. This was never done systematically, but mostly by indirection. We have recently been suffering some poor results on invertebrate tissues - and wondering if there is a perfect pH that would yield best results. Is it conceivable that a small shift in pH is critical? In particular we have been testing microwave protocols, where access across the outer cuticle may be limiting entry/exit of fluids into the intact animal. Any suggestions? I have always much more about osmolarity rather than pH. David Hall <hall@ecom.yu.edu> 21 Jun 2007

I have not done this systematically, but my impression is that the osmolarity matters and the pH is much less (if at all) important. That said, I have never done microwave, so I am not sure if that changes things. David Elliott <elliott@arizona.edu> 21 Jun 2007

There is an optimum for everything, so there is no doubt that an optimal pH exists. The question is whether it is worth it to spend much time to find it when you can be happy with a "routine" protocol. <nizets2@yahoo.com> 21 Jun 2007

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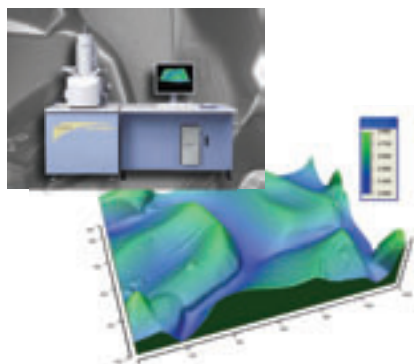
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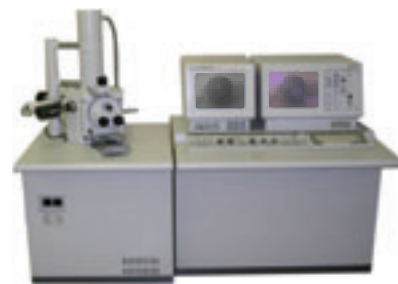


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I think that generally, the pH should be “neutral” to keep proteins soluble, but that is a fairly broad range. The buffering capacity of the fixative may be important to keep the pH “good” in use; in plants, penetration of the fixatives can be slow and the vacuolar sap is acidic so if the membrane becomes permeabilized before enough buffering is present the environment for the actual fixation in internal cells may go acidic during the critical minutes of early fixation. I think that some of the high pH fixes for plants (> pH 8) may be trying to hedge a bit, starting on the high edge so the result is neutral? I wrote to Tina a couple of days ago about the extremophiles low pH and admitted to having to fix beef samples at pH 3.5 and I was surprised that the tissue gelled very quickly at pH 3.5. Here it is important to have the pH low during fixation because the “ex-tissue” does wild things structurally and changing the pH to neutral could well alter the structure before it fixes. However cells growing @ pH3.5 may well have neutral cytoplasm - maybe someone knows. In the early 70’s, a professor I worked for was excitedly showing my glutaraldehyde-fixed, plastic-embedded material to his colleague, a famous tropical botanist. After the tropical botanist was allowed some period of study, he was asked what he thought. He thoughtfully replied, “It is all very nice, but the cytoplasm obscures all the detail”. So, optimum is relative to the need and end result? Dale Callahan <dac@research.umass.edu> 21 Jun 2007

SPECIMEN PREPARATION – UV polymerization

I am doing immuno-EM on human red blood cells (RBCs). In the past I have used cryo-sections and probing on the grid. I would like to plastic embed the cells and do antibody labeling on the grid. I used my normal protocol and embedded in Unicryl. Then my problem occurred. The Unicryl block polymerizes (UV for a few days at -10°C) just fine except in the sample pellet. I assume the UV light is being absorbed by the RBCs, and thus not penetrating the sample. So, my question is, what should I try next? David Elliot <Elliot@arizona.edu 19 Jul 2007

If the UV is the problem, you will solve it by using LR White resin with the accelerator. It will easily polymerizes at 4 degree using 1.5 microliter of accelerator for 1 ml of resin. Rachid Sougrat <sougratr@mail.nih.gov> 19 Jul 2007

Fortunately your solution is easy - cut the gelatin embedded blocks of red blood cells into very thin slices and try your embedding again. The thinner slices will give more access to the UV light and you will get the resin polymerized. I have embedded gelatin-immobilized malaria-infected red blood cells in Lowicryl (polymerizing with UV light) on many occasions so I know the trick works. Now, just don't ask me how to get rid of all the non-specific labeling over the hemoglobin! Paul Webster <pwebster@hei.org> 19 Jul 2007

SPECIMEN PREPARATION - SEM glue without carbon

I am trying to detect organic contamination on/in aluminosilicate material by SEM+EDX, so it is very important for me to eliminate any source of carbon. As the specimen holders are made of either carbon, silicon or aluminum, I am planning to use copper disks as support but I still have to fix the powder on the disks. Is there anywhere in the known universe a glue which does not contain carbon? Stephane Nizets <nizets2@yahoo.com> 26 Jul 2007

What level are you trying to detect and on what form of aluminosilicate? Are you sure the contamination is already there? Depending on the level and distribution of contamination, you may have great difficulty addressing this by EDS. If you have a uniform level of 2% or less, my gut feeling is that it would be hard to notice the change in the spectra. If the contamination is localized at high levels even though the average level is low, it should be relatively easy to search for the carbon-rich areas. You may want to test your blank stubs first to make sure you can analyze anything and show an absence of carbon. We still have diffusion and rotary pumps on our two SEMs and we always find some carbon in the spectra from hydrocarbon contamination. There are ways to reduce that buildup, but you want to establish that early. As to your original question about a carbon-free adhesive, that is a good question. I can't think of anything off hand. However, there may be some ways to cheat around the problem.

Warren Straszheim <wesaia@iastate.edu> 26 Jul 2007

You might consider another analytical method. XEDS is not the best method for this. I would suggest either XPS or better yet, TOF-SIMS. There is another method that you might consider also and that is to measure the contact angle. You could take two reference samples, one with and one without contamination, and measure the contact angle. The contact angle is a very sensitive probe of surface cleanliness. You can create a contamination-free surface by plasma cleaning or by using a germicidal lamp. Scott D. Walck <walck@southbaytech.com> 26 Jul 2007

Two carbonless glues to consider when sample heating cannot be used: Type A: Salt. If you can allow a salt to be present in your analysis, you might be able to use it like glue. If you have water that is sufficiently carbon free, and dissolve a very small amount of a permissible soluble salt into it (selecting a salt that is adequately carbon free and of known composition that doesn't interfere), voila, a type of crystalline glue. You only need a very tiny bit of salt. You only want a few nm sized salt grains between your powder grains and the substrate. Whatever mass of powder you have, scale back accordingly to calculate the amount of salt you need. It helps to do a dry run with plain water to see what volume of water you will use, and to calculate the concentration of salt you need to mix up. You can disperse your powder as a liquid suspension, or allow some of the dilute salt solution to wick in through a dispersion of dry powder on the mount. When the water evaporates, tiny salt crystals should form between the powder grains and the substrate. Remember your mixing vessel and the means by which you will apply the dilute salt water should not contribute carbon at whatever level is a problem for you. This method is not advised if you want to analyze a thickly applied powder. Type B: A weaker type of carbonless glue: Utilize near-distance electrostatic or van der Waals forces if particles are smaller than 10 microns. Add purified water (or chose another liquid that won't degrade the sample and will evaporate) to the powder. When the liquid dries, the powder particles should have resettled onto the substrate in a way that enhances the formation of van der Waals forces. The powder will stick better this way, than if you just applied the powder dry to the substrate. If you chose a very insulating liquid such as Freon or heptane, the electrically insulating properties of the liquid may tend to enhance particle agglomeration, which is not a good thing. I prefer polar liquids such as water or ethanol to help keep particles separated. This method is not advised if you want to analyze a thickly applied powder. It can work surprisingly well for fine particles covering < 5% of the surface area of the mount. I'll be interested in other replies, so please post any that are sent to you directly. Cynthia Zeissler <cynthia.zeissler@nist.gov> 26 Jul 2007

Why don't you clamp the sample onto the holder? It's fairly easy to drill and tap a small hole or two in the sample holder and make up some spring clamps to hold the sample down. Larry Stoter <larry@cymru666.plus.com> 28 Jul 2007

Still, why do you need to glue at all? Can't you just put some of the sample on the mount? Larry Stoter <larry@cymru666.plus.com> 29 Jul 2007

Well, first of all, I will definitely try by resuspending in water and letting dry, but still the holder will be whether in Si, C or Al, which is a bad choice in all cases. Second, I already tried with TEMPFIX (it is a resin which “melts” at 100°C but is hard at room temperature) and even if I blew on the particles before coating them and putting them in the SEM some particles disappear where they are scanned! Apparently I have to immobilize them otherwise I will contaminate the SEM. Stephane Nizets <nizets2@yahoo.com> 31 Jul 2007

Unless you plan on putting large amounts into the SEM, I seriously doubt that contamination is a real issue. Mill a small hole ~1 mm deep into the mount and put the powder in that. If really necessary, cover with a thin foil TEM aperture. If you use one with ~100 nm aperture hole, that will keep the vast majority of the powder in place. How about some Ti as a suitable support? Larry Stoter <larry@cymru666.plus.com> 31 Jul 2007

TEM – calibration

Recently I was in a discussion with another microscopist and we were

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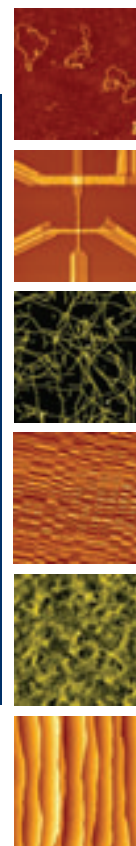
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NETNOTES

wondering what most TEM users/TEM labs use to calibrate their instrument. To settle our bet, (grand prize is the extensive bragging rights of being one up) let me pose the questions to the list: "Do you use a calibration device to confirm the scope's magnification? And if so, what do you use?" I use a 2160 line grating. Frank Karl <frank.karl@degussa.com> 31 Jul 2007

I use the cross-grating grid as well, but I don't need high precision at high magnifications. I suppose for ultra-high magnification something like crystal lattices should be used. I once discussed about the use of colloidal gold to calibrate high magnification. My colleague (an experienced TEMist) told me that the size distribution of colloidal gold particles had a Gaussian distribution, so it was a bad idea in the end. Stephane Nizets <nizets2@yahoo.com> 31 Jul 2007

We use a line grating. We do not calibrate the TEM, we calibrate the digital camera (much easier than doing the CM12). David Elliott <elliott@arizona.edu> 31 Jul 2007

For really high magnification one can use the crystal lattice, which is visible in a sizable fraction of the colloids. I agree that trying to use the diameters of the particles is not suitable. Bill Tivol <tivol@caltech.edu> 31 Jul 2007

We use the MAG*I*CAL standard, probably the most accurate standard ever made for TEM. I believe it is not NIST certified and traceable. I am happy to report that our TEM's (Hitachi 7100 STEM and 7650) fall within 2-5% of the stated magnification. Most scopes are guaranteed to be within 10%. John Bozzola <bozzola@siu.edu> 31 Jul 2007

I use a III-V superlattice, which has nice contrast (essentially black/white) in (002) dark field. The period of such a superlattice can be determined to 1 part in 10⁵ using X-ray diffraction. There are two periods, so it covers low magnification (down to about 5000X) and higher mag (up to a few 100000 X). Above that I use lattice fringes. It's possible to calibrate to better than 0.25% with this standard - although I find the magnification

varies by about 0.5% from day to day so unless it's critical it's not worth putting too much effort into getting a super accurate calibration. Basically this is my version of the "mag-i-cal" standard which is based on SiGe - although it's not NIST certified it's easier to work with and I'm happy with the X-ray measurements used to obtain the period. The difference between indicated and real magnification is pretty small on the Jeol 2011 I used most recently (within 3%) but an older 120CX was consistently about 10% off. Richard Beanland <contact@integrityscientific.com> 31 Jul 2007

I do it according to Philips' instruction, 20 to 1500x - 1500 mesh grid 1500 to 100Kx - grating replica (2160 lines/mm) >100Kx - either extrapolate from calibrated lower magnification images or use lattice spacing. On our Philips CM100, the accuracy of magnification is listed to be within 5% of that indicated on the display. For practical purpose, I do the calibration with respect to our digital camera and it is indeed within 5% for the 1500 to 100kx range. I have not checked the other ranges. W. Chan <wpchan@u.washington.edu> 31 Jul 2007

The votes are in and here's the partial summary of what people use to calibrate their TEM: My informal survey is based on 24 responses: 63% (14 people) use a grating replica, 68% (15 people) use crystal spacing, 26% of the respondents indicated they use a commercial product called Mag*I*Cal. For more details and a little verbiage visit Microscopy Society of Northeastern Ohio Blog at: <http://www.msneo.org/2007/08/scales-and-distance.html>. You can also find what I think is the most unique TEM calibration I've ever read. Frank Karl <frank.karl@degussa.com> 03 Aug 2007

TEM - 120 Kev vs 200 Kev instruments

200Kev scope users: We are trying to evaluate 120Kev TEM's vs. 200Kev TEM's with LaB6 guns. Our biggest concerns come in routine usability, and I am hoping to hear any comments on any of the following questions we have. We handle a wide range of samples: biological thin sections, organic macromolecules, geologic particles, geologic thin sections, crystals, thin films, nano-

particles, organic and non-organic materials with a wide range of techniques (BF, DF, Diffraction, EDS, EELS, including lattice fringe imaging, STEM and tomography secondarily). In balancing contrast, resolution, and beam damage we really expect to perform a majority of our imaging between 80KeV and 120KeV (or 150KeV). However, the option of higher voltages up to 200KeV, for higher resolution imaging is something we are considering. So we are looking at both 120 and 200 KeV scopes. And yes, we will test as many samples on any scope as we can but a couple of days worth of playing with a handful of samples does not compare to years worth of experience. (1) It seems that most 200Kev scopes are set to 200Kev and left there - presumably because it eases alignments, voltage stability, etc. when pushing for ultimate resolution. Is this true? Yes, all the manufacturers list voltage ranges the scope can be operated at, but does anyone really use anything else? With the digitizing of the scope controls and storage of multiple alignment setting, alignment at different voltages (theoretically) should be fairly straight forward but is it really?(2) Is 200KeV worth having if the majority of the work does not require 200KeV? Not having personally worked much at 200KeV, Does loss of contrast and beam damage at 200KeV limit usability when imaging other than atomic / lattice level?(3) If the majority of the scope usage is 80 - 140 KeV is the cost of a 200KeV instrument worth it in terms of initial cost, and maintenance? Even if the only option tops off at 120KeV. We are all aware of instances of: "If you can at all afford it get, even if you won't use it regularly, because . . ." is this one of those? (4) A few years ago the list had a discussion of W vs. LaB6, vs. FEG/Schottky for SEM's and the consensus basically was that LaB6 was not worth the cost for SEM. (Either FEG if affordable or tungsten with better bells & whistles). Is LaB6 worth it in terms of TEM? (5) What more important issue have we neglected in our naivete? Boy, wouldn't a 150KeV Schottky-FEG be a nice compromise? Richard Edelmann <edelmare@muohio.edu> 19 Jun 2007

The first misconception is that 200 kV causes more beam damage than 100 kV. It causes less. More beam goes through the sample without dumping its energy, therefore less heat is dumped into the sample and there is less beam damage. Usable viewing area and diffraction is much improved at 200 kV. I have a 200 kV W-filament TEM. I use it at mainly 100 kV and 200 kV: 100 kV for light-element samples to get the contrast and 200 kV for better resolution and better penetration, therefore larger viewing area in metal samples. I have also looked at thick, unstained sections in botanical samples (0.5 micron, 0.75 micron and 1.0 micron thick) at 200 kV. If I were to replace this very old TEM, it would be with a 200 kV or 300 kV LaB6. The extra brightness is worth the small extra cost, the FEG in TEM is very expensive. I would get the STEM option so I could do EDS and EDS mapping. My TEM has five steps of acceleration voltage: 75, 100, 125, 150, 175 and 200 kV. All can be independently aligned and the alignment stored. Then, changing kV is simply pushing the button. In the new scopes, you can store the alignment on a CD or hard drive, so you can recover if one of your users screws things up horribly. I envy you the chance; I cannot get the people here to go seriously after a new TEM. Mary Fletcher (nee Mager) <maryflet@interchange.ubc.ca> 19 Jun 2007

I well remember my days as a TEM demonstrator trying to persuade users, often unsuccessfully, that going to higher kV really did reduce damage. However, while for many samples, higher kV does result in reduced thermal damage, for some samples knock-on damage begins at surprisingly low thresholds and this does increase as the kV increases. A particularly topical example is carbon nanotubes, where there is evidence that knock-on damage begins at ~80 kV. You also need to remember that with digital CCD camera systems, image contrast is much less dependent on kV, so with a good digital camera, you can get both improved resolution and good image contrast by operating at 200 kV. LaB6 is definitely worth using on a TEM. Unlike SEMs, there is generally no additional cost apart from the filaments themselves, since the gun vacuum levels on modern instruments should be more than sufficient for LaB6. At higher magnifications, the combination of greater brightness and smaller spot sizes usually means that the images are brighter. In addition, filament lifetimes should be significantly longer. LaB6 filaments also give somewhat better beam coherence, which in practi-

cal terms results in better contrast in high resolution images. My feeling is that really the only reason to go for 100/120 KV is that the budget isn't available for 200 kV. Moreover, while you can always turn the kV down on a 200 kV instrument, you cannot turn it up on a 100/120 kV instrument! Larry Stoter <larry@cymru666.plus.com> 19 Jun 2007

In a universal applications environment the only good reason to buy 120kV vs 200kV TEM is lack of money. Krassimir N. Bozhilov <bozhilov@ucr.edu> 19 Jun 2007

Krassimir: Then you have no problem running any KeV at any time in your TEM(s)? Richard E. Edelmann <edelmare@muohio.edu> 20 Jun 2007

There is no problem running at any kV one desires. Furthermore, the quality of images at higher voltages is far superior due to the better coherence of the el. beam for the LaB6 cathode and decreased inelastic scattering compared to tungsten and low voltage. Also the need of lower voltage for improved contrast is questionable if the system is equipped with digital imaging capability. As long as resolution is there contrast can be improved quite significantly just by post-acquisition processing. Radiolysis is the main problem causing specimen damage at voltages less than 300 kV and this increases with decreasing accelerating voltage. That is another advantage to operate at higher voltages. The only drawback for having higher voltage instrument with LaB6 is the stricter vacuum requirements. For many life science applications this may cause some slowdown since pumping times will be longer during sample exchange. This can be resolved by obtaining multiple-grid specimen holders. I would even suggest if funding is not a problem to go for a 300 kV instrument, provided you expect to do substantial work in materials science and geology. Krassimir N. Bozhilov <bozhilov@ucr.edu> 20 Jun 2007

I will join the opinion of previous posters of the list. A LaB6 at 200keV just offers more flexibility for no disadvantages except the cost. I don't know the difference with W gun. With my TEM I can even work at 1 keV if I want to. And I can store all the settings I want at any keV or any magnification I choose. I would add that if you plan to work on nanoparticles you will probably appreciate the additional comfort of a LaB6 working at 200keV at high magnification. Stephane Nizets <nizets2@yahoo.com> 20 Jun 2007

Please let me contribute one aspect to this thread that has not been considered: unless you purchase a TEM with constant power lenses, and I am not aware of any other than the FEI Titan, you will have a lengthy period of thermal instability following an accelerating voltage change because the lens currents change significantly. Dropping accelerating voltage can result in over-cooling the column, which can result in condensation, vacuum leaks and thermal drift, even if the water flow is adjusted. Raising the accelerating voltage after an extended period of low-voltage operation can result in a need to condition the accelerator to avoid HT instability. Those are some of the other reasons that TEMs generally run at their rated high voltage. John Mardinly <john.mardinly@intel.com> 20 Jun 2007

John, what you are pointing out is true in principle and in theory and it could affect seriously only dedicated HR TEM instruments. I have experience with a Philips/FEI EM, CM and Tecnai TEMs. As an example I will point out to our CM300 which for the past over 10 years has been operated routinely between 100, 200 and 300 kV. This machine has theoretical point resolution of 0.23 nm and we have never encountered problems with obtaining the specs for resolution and stability even when we had to switch between different voltages within several hours time span. If you have a FEG instrument with objective lenses with small coefficient for spherical aberration then minor instabilities are prone to cause considerable consequences but on a W or LaB6 instrument with relatively large gap between the objective polepieces the problems are negligible. In essence what I want to say is that if one needs to operate an instrument at the highest possible resolution and stability then operating at the maximum voltage is probably what should be expected but for a machine designed and dedicated for universal application there are no serious problems with using range of voltages specifically with the FEI twin lens instrument which I have extended experience. I suspect that this would be true for JEOL, Hitachi

and Zeiss but cannot confirm it from my own experience. Krassimir N. Bozhilov <bozhilov@ucr.edu> 21 Jun 2007

Back at Argonne National Lab, we had a JEOL 4000 that was kept at 400kV to high resolution work. It was aligned by the service engineers at 100, 200, 300 and 400, so recall of alignments was a push of a few buttons. At lower voltages the lenses run less power and are much cooler but generally did not affect resolution for most work. To run at 400kV for HREM imaging the TEM had to sit overnight to thermally equilibrate. When needed, it ran at 100, 200kV. If someone really needs high resolution stability, set the HT to the setting wanted the night before; this is easily handled by indicating the voltage wanted when someone signs up for their time. If a user really wants resolution they will remember to indicate the desired voltage. Condensation and vacuum leaks were not a problem. Roseann Csencsits <rcsencsits@lbl.gov> 21 Jun 2007

TEM – cause of specimen damage

I have enjoyed reading the discussion over the past few days and hope you don't mind if I ask a question related to the whole 200 kV versus lower kV instrument discussion. I recently had discussion with a colleague concerning specimen damage in a TEM at 200 kV versus lower kV and told them that 200 kV machines actually do less damage to samples due to faster moving electrons and therefore lower interaction times (something someone echoed here on the list). What we couldn't get to the bottom of was the reason that, when investigating carbon nanotubes, the person in question said she saw more damage in a 200 kV machine that at lower kV, suggesting there was less time to image the tubes at higher kV. My question is this, is there something particular to carbon nanotubes in the TEM that makes their radiation damage behavior contrary to the established rules? Christopher DJ Parmenter <christopher.parmenter@warwick.ac.uk> 26 Jun 2007

There are (at least) three mechanisms of specimen damage in the EM. In order of their occurrence with kV: 1.) Radiolysis - the energy of the electrons breaks the molecular bonds in materials and disrupts the



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structure. This is the principle effect in organic materials and what most people were talking about previously. The cross section for this effect decreases with increasing Energy. Hence the motivation to increase the kV. 2.) Sputtering - the energy of the electron is sufficient to remove an atom from the surface of the material (most frequently the beam exit surface of the sample). The cross-section for this has a threshold and then increases with energy. Depending upon the cleanliness of your microscope you may or may not see this effect. Hydrocarbon contamination does not sputter strongly and thus acts like "glue" holding the surface atoms of your real sample in place, so to speak. 3.) Displacement - the energy of the electron is sufficient to knock an atom out of its location in a crystalline lattice inside of your sample. This is the highest energy process, and is the bane of materials scientists as they go to higher voltage instruments to look at thicker samples, or when high voltage is used for high resolution. This effect creates vacancies, interstitials, etc., which are usually mobile. BTW, this is sometimes called Knock-on damage. All of these effects are a function of atomic number, bond strength, lattice strength, dose, dose rate and kinetic energy of the electron beam. Each will manifest itself at different energies for different materials. Because of all of these factors for each material there is an optimum voltage to operate at, which is at the local minimum created by the intersection of these 3 processes. The important point is that it is never zero in a typical TEM environment. In your carbon nanotubes you are likely seeing #3. The simple experiment to do is, keeping the illumination conditions approximately constant, drop the kV in your instrument and observe the nano-tubes. You will find a voltage at which the damage as a function of time is minimized. My guess is that this will be ~ 90-100 kV. Because of the threshold, and the fact that it takes time for damage to accumulate, you can work at higher voltages for short periods of time before defects accumulate sufficiently to disrupt a given materials structure. Of course if your working with single walled carbon nanotubes this won't be very long. I'll dig up a few papers on sputtering/displacement damage that colleagues and I worked on a sometime ago on this and put it on the following WWW site later this morning if you are interested in reading a bit more: <http://tpm.amc.anl.gov/Lectures>. There should be references to the sputtering/displacement damage calculations therein. For radiolysis you will have to go to the life science EM literature or one of our life science colleagues will post a message. Nestor Zaluzec <zaluzec@microscopy.com> 26 Jun 2007

TEM & SEM terminology - kV or keV?

In TEM the high-tension (HT) value is usually expressed in kV whereas for SEM it is usually expressed in keV. Being a biologist, I don't easily comprehend the difference between the 2 units. In TEM the HT value represents the real tension between the anode and the cathode. I expect that is the same in SEM, so why add an "e" in-between? Does it change anything, except the unecological use of extra amount of ink? Stephane Nizets <nizets2@yahoo.com> 29 Jun 2007

HT is a voltage and therefore always has the unit V(olt) or kV for kilo Volt. keV stands for kilo electronvolt, which is a (non-SI) energy-unit. That is something completely different. To get from voltage to energy you have to multiply with the charge of the particle traveling through the potential-difference (or voltage). 1 eV is the kinetic energy of an electron that has been accelerated by a voltage of 1 V. Just to make things clear: This is independent of the electron's mass. It only depends on charge and voltage! Philip Koeck <Philip.Koeck@biosci.ki.se> 29 Jun 2007

kV is kilovolts, an electrical potential (SI units). It is the electric potential between the anode and cathode. "keV" is kilo-electron-volts, a unit of energy (doesn't matter if kinetic or potential). It represents the energy of a particle having a unit (electron) charge in a 1 kV field (or its kinetic energy after passing through a 1 kV field). Although not strictly SI, it is considered a "unit accepted for use with SI"* It has nothing to do with the particle's mass. One can use either to describe the operating conditions of your SEM or TEM interchangeably, since we're always accelerating electrons. For EDS or EELS spectra, the unit is always keV or eV (unless an SI purist prefers zeptojoules) since in the case of EDS one is referring to the energy of an

x-ray (chargeless and massless!), and in EELS one is measuring energy transfer by an electron.* See R. A. Nelson, "Guide for Metric Practice," Physics Today BG13 (1997) <fortner@cmt.anl.gov> 29 Jun 2007

ELECTRON MICROPROBE - carbon coater

We are in the process of setting up a probe lab and are in search of a carbon coater. I would appreciate advice about carbon fiber versus rod and turbo pumped versus non-turbo for microprobe analysis of polished thin sections. Ellen Metzger <metzger@geosun.sjsu.edu> 29 Jun 2007

Our Electron Microprobe Lab has a JEOL JEE-400 Vacuum Evaporator, mid-1990s vintage. It uses sharpened carbon rods and has a compound rotary-diffusion vacuum system. The vacuum system valves are all manual (no electronic automation), and the threat of cleaning burnt diffusion-pump oil instills a healthy sense of fear in students. A turbo pump will increase the cost of your coater by about 50% (last time that I priced them), and it will be faster but not necessarily any better. Diffusion pumps are hardy, and after 12 years or so, I think we are on our third rotary pump (although we probably could have repaired the broken ones if we were so inclined). In our experience with samples coated in other facilities, the fiber-type carbon coaters generally do not seem to apply as smooth a coat as our rod-type coater. I usually insist that samples going in our microprobe be coated in our lab, not elsewhere. This, though, is based only on anecdotal evidence and our experiences, not any systemic study. Ellery E. Frahm <frah0010@umn.edu> 29 Jun 2007

Turbo pumped systems pump faster. They pump to a lower pressure than rotary pump only systems (i.e. table top models). Lower pressure equals finer coating grain size. Fiber (rope, cord, etc.) is a quick and easy carbon coat, and can get away with higher pressures, but it is rougher, and can leave "chunks" of fiber on sample. Not particularly controllable as to thickness (use more fibers vs. fewer fibers). Rods systems tend to be finer coating, more controllable as to "thickness" (evaporate more or less of the rod). If you are thinking about EBSD work in the future, and might wish to use a very thin carbon coat (yes, before someone yells out, it will obscure some of the signal). The control of the rod systems is needed. I have not compared fiber vs. rod for electron microprobe work on polished sections. We use a full blown Denton vacuum evaporator (very fine, very versatile, very slow), and I really wish I could get a turbo pumped table top model for most SEM work. Richard E. Edelmann <edelmare@muohio.edu> 29 Jun 2007

I've got to take issue with this disparaging information about carbon string evaporation. Carbon string gets criticized in the way given below, and I think that some of this comes from using it with rotary pumped vacuum levels, but possibly also the method of evaporation; the method usually given is "flash evaporation". We pump down to at least 2×10^{-5} Torr (~ 0.0027 Pa) and evaporate in a series of pulses that bring the string to just white-hot and then switch off for a couple of seconds, repeating until the string "sparkles" the final remaining fibers. I have found the method highly repeatable and easy to arrive at a repeatable thickness. Call this "pulsed evaporation"? We use two different systems, neither designed for carbon string. One is a Balzers BA080T turbo-pumped unit, and the other is a Kinney KDP-2A (circa 1965) ODP pumped system. For the Kinney, easier to explain, I use a short 1.25 cm spacing tungsten boat holder and just lightly clamp the carbon string where the boat would normally go - 1.25 cm exposed length. This is set at a distance chosen for the desired thickness, ~ 5.5 - 8 cm typical. The system is pumped down to below 1×10^{-4} Torr (2×10^{-5} Torr typically) and the string is degassed by slowly raising the voltage to give a medium bright orange glow - much faster vacuum recovery than with rods - and then switched off. The Variac (controls the input to the power transformer) is then set for $\sim 40\%$ (without power applied!). This is 40% of max, max being 24VAC nominal of the power transformer secondary. The switch is flipped on (view through dark glass, and as soon as it seems to have risen to white and stable temperature (~ 0.5 sec), switch off, repeating this cycle after a few seconds until burnout. This method was chosen to allow the carbon to reach evaporation temperature, but not send it all off at once. The radiation heating is minimized and spread over

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time. I coat the "back side" of Formvar that is placed over 5mm holes (for the method of picking up sections with a 1x2 mm slot grid and setting the grid on the upper non-carbon-coated side of the filmed area) and I see almost no damaged films using this method. We had much trouble trying this with rods (1/8"), turned down to 1 mm diameter x 2 mm long tips). We use a bit of very white paper stock stuck under the mica, etc., as an indicator of thickness by color; looking through my notebook, it is highly repeatable. In making adjustments to vary thickness, keep in mind that the thickness is inversely proportional to the square of the distance; a small change in distance has a substantial effect on the film thickness. By setting the voltage and pulsing time appropriately, it should be possible to get good coverage of rotating samples as well. Different yarns can also be used, selecting a voltage to give similar characteristics to the evaporation. It seems that the SPI #11433 thread just comes to a stable white heat in ~0.5 sec, and that will vary for different product diameters. Most EM supply vendors carry a selection of thicknesses. The "thread" mentioned above gives a nice balance between thickness and heating. Thicker thread could be used at longer distances. One could experiment with different lengths as well; we have good success with the 1.25 cm length. Dale Callahan <dac@research.umass.edu> 29 Jun 2007

SEM/EDX - thin film thickness measurement

I am trying to measure the thickness of Mo film (about 100nm) on a Si substrate by SEM/EDX and Wincasino simulation. From my understanding, Wincasino can simulate a calibration curve of film thickness vs. the x-ray peak ratio of Mo/Si with the same parameters used in experiment (beam energy, TOA, etc.). The real thickness can be located on the calibration curve based on the experimentally measured Mo/Si ratio. It is supposed to be an accurate method but I have two questions: 1. Is this method sensitive to the experiment settings (beam energy) and how accurate is it? 2. In Wincasino, which x-ray intensity can be used for peak ratio calculation, absorbed or non-absorbed

intensity, or the difference of them? I tried some measurements but they are not promising. Please also correct me if the procedure I described is wrong. Xiaohu Tang <xiaohutang@gmail.com> 10 Aug 2007

If you only need thickness (not composition), this thickness is perfect for x-ray reflectivity measurements and is very accurate because it looks at the constructive/destructive interference pattern. John Donovan <donovan@uoregon.edu> 10 Aug 2007

Be careful interpreting the results. Microanalytical experiments like this measure the mass-thickness not the thickness. If you believe you know the density of your film then you are ok and you can then calculate the thickness. If the film density is different from your assumed density (often bulk densities) then you may run into problems. Nicholas Ritchie <nicholas.ritchie@nist.gov> 10 Aug 2007

EDX signal saturation I am having a too strong signal in EDX (in SEM). The dead time is usually between 70-80%. I wondered how I could reduce the signal without changing the high-tension (HT). Changing the size of the beam is one way. Would it be wise to move the stage in the Z axis to be sub-optimal with respect to the EDX detector? Stephane Nizets <nizets2@yahoo.com> 09 Aug 2007

It is not a good idea to move the Z axis of the SEM stage for EDX, this will upset the geometry of the beam-detector system and affect quantitation. There are three possibilities: get an SDD detector, which is much more tolerant of high beam currents, use a higher condenser lens setting (smaller beam spot size) or a smaller final aperture, if your SEM has a movable final aperture. You could also move the EDX detector back along its track (retract it), which will change the solid angle but not the geometry. You may have to re-enter the geometry in the EDX setup if you do that. If you change the HT of the SEM, you may not detect all the elements. Mary Fletcher (nee Mager) <maryflet@interchange.ubc.ca> 09 Aug 2007



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