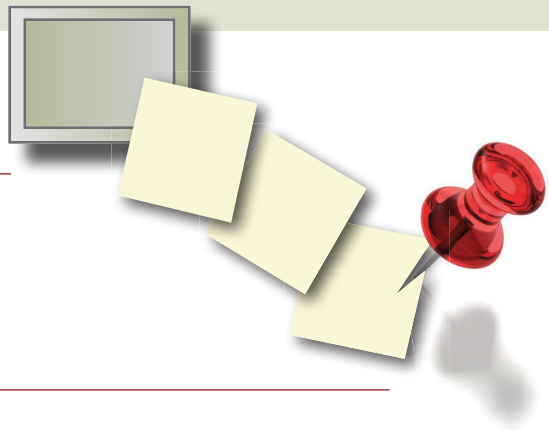
NetNotes



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Selected postings are from recent discussion threads included in the Microscopy (http://www.microscopy.com), Confocal Microscopy (https://lists.umn.edu/cgi-bin/wa?A0=confocalmicroscopy), and 3DEM (https://mail.ncmir.ucsd.edu/mailman/listinfo/3dem) listservers. Postings may have been edited to conserve space or for clarity. Complete listings and subscription information can be found at the above websites.

Microscope Incubation Chambers: Cage or Stage?

Confocal Listserver

In common with many of you, I manage a facility where users want to use many different model organisms. We mostly have cage-type incubation systems on our microscopes, which is less than ideal when one user wants the microscope at 26°C, and the next user wants it at 37°C. I have found that cage-type incubators do have the advantage of suppressing temperature fluctuation caused by thermostat control AC, so it's good to have them around the machine. However, I would like to know whether there are stage-type incubation systems I could use inside the chambers that would allow us to quickly switch between imaging specimens at 20, 26 and 37°C (possibly below ambient, so with cooling) and also provide a reliable way of maintaining focus with high NA immersion lenses. I have used objective lens heaters before and some (but not all) have been very good, but are there any that won't clash with the stage or microscope when the nose piece is rotated? I'd like to leave them in position rather than take them off all the time. Any suggestions would be most welcome. Thanks, ***Andrew Vaughan andrew.vaughan@ucl.ac.uk***

What kind of microscope did you use? I think it is an inverted one, but what were the conditions? Pascal Weber pascal.weber@univ-amu.fr

The microscopes are inverted. The rooms are just standard microscope laboratories. The conditions vary according to the model organism. Some users have mammalian cells which need 37°C, some have yeast which are happy around 20°C and some people are using *Drosophila* tissue at 26°C. Andrew Vaughan andrew.vaughan@ucl.ac.uk

We use hotbox-type boxes too, but we have an LSM 710 with a Pecon stagetop incubation system for Ibidi microwell plates that rapidly equalizes to the required temperature. It has excellent XY repositioning and the Zen autofocus works well for slower time-lapse experiments, though Definite focus/Perfect focus/ZDC type hardware autofocusing might be a faster means of getting back to a stable Z position. Hotboxes are great, but the expansion and contraction time for a stable microscope setup under different conditions is tedious. The stagetop design works reasonably well for our needs. All the best. Darran Clements dkc25@cam.ac.uk

We carry a brand-new product by a startup company called Linnowave which is out of the Max-Planck Institute in Erlangen, Germany. Their novel solution is to have the actual cover slip be the heating and measuring element using transparent, conductive coatings. It measures and generates heat where you need it: near the focal plane at the coverslip surface! It comes as a 18×18mm coverslip size and is available also with a PDMS liquid reservoir. All you need is a humidity chamber which can be equilibrated much faster than conventional heating elements based on equilibration of an entire sample system, including objective. Since the temperature control with the Linnowave VAHEAT is targeted at the very volume where your focus is it can adapt within seconds. For more information please visit https://linnowave.com/ and https://shop.boselec.com/products/vaheat-microscope-temperature-control. Jens Breffke jens@boselec.com

Having looked at the literature, I am not clear where the temperature is measured. Is an integrated measurement across the whole coverslip being made? If so, any immersion objective will act as a heat sink and reduce the local temperature for the actual cells being imaged, but this will contribute little to an overall temperature measurement - and not be compensated. If compensated and the heating increased, then cells at a distance from the objective will be heated above 37°C. I have a strong preference for heating everything to 37°C so local gradients around the cells/objective cease to be an issue. Have a look at J Microscopy 210 (2003) 131-137. We found some wonderful distortions when a slow Z-series had a similar timescale to the room temperature oscillations. Jeremy Adler jeremy.adler@igp.uu.se

Let me help to clarify: Our VAHEAT system is specifically designed to compensate the effects you were mentioning: 1) Local heat sink in the FOV and 2) thermal drift. We have a highly sensitive temperature probe sitting in the FOV that enables fast feedback to adjust for temperature variations in the environment. The specific geometrical arrangement of the heating element, temperature probe and the immersion objective insure a homogeneous temperature distribution in the FOV. Additionally, the heat load injected into the objective via the immersion oil is kept to a minimum and the quality of the optical imaging system can be maintained, even at elevated temperatures (up to 80°C). This allows for fast, reproducible temperature changes and long-term temperature stability without warming the whole microscope to the desired temperature (which takes several minutes to hours and usually doesn't improve on the temperature accuracy). All in all VAHEAT can, thus, be combined with various highly sensitive measurements such as AFM/confocal, STORM or PALM, PAINT, STED, TIRF. Pierre Türschmann pierre@linnowave.com

Here is how I address the micro-environmental issues you described. I agree the box approach does reduce the thermal expansion of the whole scope that occurs due to room temp variations. However, in that you describe the need to use immersion lenses for and during temperature transitions, I recommend keeping the thermal mass of the specimen containment structure to a minimum and applying or removing heat to that immediate area in the most efficient

means possible. Waiting for self-equilibration takes a looooong time. My company, Bioptechs, has developed micro-environmental control systems for both steady state and quick thermal ramping applications such as gene expression experiments. Therefore, I believe we can precisely meet your needs. In addition to typical fixed physiological temperatures, our systems are being used in the temperature ranges of, 15°C to 25°C in 20 seconds, 4°C to 40°C in 3 minutes, and 15°C to 60°C in 2 minutes while maintaining stability in the Z-axis within our FCS2 flow cell. If an actual laminar flow cell is not needed, we have a number of other products and technologies for fast and efficient thermal control as well as Z-axis stability to choose from, including a coverslip-bottomed dish with intrinsic temperature control at the coverslip. Temperature control for the objective is a specialty of ours. Our patented Objective Heater incorporates the objective temperature at the specimen plane in the control loop and compensates for the heat-sinking effect of the nosepiece. It has a detachable cable from the heater so that you can rotate the nosepiece. If you need to obtain temperatures below ambient, we have products to do that as well. Special note, we developed a thermal control system used on all our products that takes the specimen from initial to target temperature typically in seconds then steady thereafter and it does not induce the variations typical with PID. Setting up a micro-environmental control system for imaging is not trivial. Dan Focht dan@bioptechs.com

Multiple-Position Long-Term Time-Lapse Imaging with Oil Immersion Lens

Confocal Listserver

One of our users is doing long-term time-lapse imaging in multi-well plates with reflection based autofocus. The sample is quite flat, so an oil immersion lens seemed the best choice in terms of light collection efficiency and image quality. Our first run worked well. However, during the second experiment the focusing failed quite early during the time-lapse and the sample remained out of focus for most of the experiment. I suspect that an air bubble in the immersion oil could have interfered with the focusing and because of high viscosity of the oil, a bubble can persist there for quite some time. I'm wondering if the hypothesis of the bubble is correct. How likely is such an incident to happen - whether we were particularly lucky in the first experiment or particularly unlucky in the second? What is your experience with the use of oil immersion in such experiments? ***Radek Machan radek.machan@ntu.edu.sg***

If the sample is in water, I would use a water objective and have oil with RI=1.33 as the immersion medium. You will get better image quality since your sample is very likely to be thicker than 10µm. On top of that the oil with low RI is less viscous so less likely to have bubbles. I would apply the oil on the objective with a rod, not with a bottle that needs to be flipped and squeezed. You will need to check that the autofocus works with the water objective. Depending on how many weeks you are imaging, another reason for the loss of focus might be that the oil was spread too thin on the plate. Sylvie Le Guyader sylvie.le.guyader@ki.se

We have quite some experience with multi-well plates and immersion oil objective lenses. An air bubble could have caused the auto focus to have failed, but excess or too little oil can also cause the auto-focus system to fail as the oil runs off the lens or is spread too thin shortly after starting acquisition. Most microscope vendors include the auto-focus status in the raw file metadata for each timepoint and position. You could check back and see when the auto-focus system failed by looking at the status code to trace if the focus or another malfunction occurred. Here are some tips that might prevent this issue in the future: 1) We use a folded piece of lens paper to distribute a thin oil layer on the glass and then remove the lens paper on a part of the glass you will not image (air bubbles are introduced when lifting the paper off the glass!); 2) Move around to all xy positions a few times to evenly smear the immersion oil and then start setting up auto-focus; 3) use 96-well or 348-well plates if possible and space samples close together, also this allows more conditions to be imaged simultaneously; 4) slow the xy-stage movement down if hardware supports this and if it doesn't impact the experimental setup too much. Timo E.F. Kuijt t.kuijt@hubrecht.eu

I think your hypothesis is very likely. Additionally, what could have happened is that during the stage movement the oil was dragged and did not cover the top of the lens during acquisition. Or the focal plane across the plate plane was not the same. In general, multipoint time-lapse with immersion oil is very challenging because of the issues you bring up. Plates aren't completely flat and slight changes in Z will either make the experiment fail or damage the lens. Things you could do is to reduce the speed of the stage, "paint" immersion oil on the glass (this will make a huge mess and can potentially damage the stage if oil gets inside), or determine a map of Z positions throughout the plate so that the nosepiece can adjust for the different focal plane and hopefully damage the lens less. Paula De La Milagrosa paula_monterollopis@hms.harvard.edu

I do that experiment quite a lot and yes, bubbles have been the bane of my existence. Before I start an experiment, I always do a quick bubble test - just move the stage around and look carefully at the meniscus. You can spot bubbles because they move like bearings, at about half the speed the stage is moving. If you spot a bubble, take the

imaging chamber off and clean everything thoroughly, then oil it and check again. You can sometimes remove a bubble with the edge of a lens tissue but more often that just makes things worse. Keep in mind that it doesn't have to be a bubble - sometimes these systems just lose their lock and never get it back. For example, if you don't quite have enough oil then you can easily break the focus lock partway through an experiment. Also, note that if your system has the option to choose between continuous and point-by-point mode, continuous mode is a lot more likely to scotch the entire experiment if it momentarily loses a lock. For this reason, I generally use point-by-point mode for long-term multipoint time lapse experiments, and continuous mode for higher-speed and short-term acquisitions.

Another non-bubble-related problem has to do with how far the stage moves between points. Most systems default their stage to move very fast between multi-points to save time but moving fast over a long distance can break autofocus lock. Think about this when you set up your multi-points - try to minimize how far the stage moves from point to point and keep an eye on how far it must move between the last first points. If necessary, create 'dummy' positions between distant points so that the stage can pause and re-calibrate. Nikon (no commercial interest) has a great feature that automatically re-orders the multi-points to minimize stage travel; with other scope makers you just have to keep all this in mind while setting up the experiment. Slowing down the stage movement during multi-point helps a lot, especially with the continuous focus setting.

If you are using an infrared beam autofocus (Nikon, Leica, etc.) then I don't recommend 1.33 RI water-matched oil. Those autofocus implementations need a refractive index mismatch at the coverslip, so they often struggle to get and hold a lock with water or water-mimicking oil immersion. I agree with others that it's a good idea to thoroughly spread the oil around before starting the experiment. Also, use an objective heating collar and give the whole system an hour or two to thermally stabilize before you leave it unattended. Timothy Feinstein tnf8@pitt.edu

In an exact set up as yours, I have encountered similar problems. To overcome the issues I did the following: 1) Before putting the multi-well chamber on to the stage, I made sure that the microscope was well-equilibrated with the incubation chamber at 37°C. 2) Coat the bottom part of the glass coverslip with a film of oil by rolling the cylinder part of the glass tip applicator. In my experience, using a glass tip applicator reduces bubbles. 3) Put oil on the objective and set the plate on the stage. 4) Make contact between the objective and the plate and bring the cells into focus. 5) Allow the plate to equilibrate at 37°C for 30 minutes. 6) Select all regions of interest in the software and set the perfect focus (reflection-based focus value) for each point. Because of a slight slant, as I move from well to well the focus will change. Not waiting for 30 minutes to equilibrate also results in incorrect reflection-based focus values for the entirety of the experiment. Move the plate through this path manually a couple of times to make sure that oil is evenly spread out and there is no possibility of a bubble forming later due to insufficient oil. Note: Initially, my experiments failed because the thermal drift with only the stage top incubator was overwhelming for a reflection-based system to manage after a few hours. Using both the stage top incubator and an incubator that encloses an entire microscope, I have done time-lapse for up to 20 hours. Gaurav Joshi gnjoshi@emory.edu

Imaging of Organoids

Confocal Listserver

I have a user who would like to image live organoids via confocal/multiphoton without jeopardizing the sample. Has anyone figured out a material in which the organoid can be placed that would eliminate movement/drift during imaging, without damaging it so the sample can go back into culture afterwards? So far, the papers I've looked at on this topic cryoembed them at a certain time point for immunohistochemistry. My user's lab has samples which are expressing GFP and RFP and they would like to image them in vivo. Placing them in a mix of matrigel and culture media would slow them down temporarily, but in my experience, matrigel attenuates the signal. We have a multiphoton system on an inverted Zeiss platform (LSM 710). We can image using an objective inverter and dipping lenses, or from the bottom through a cover glass dish. Since we are working remotely, I was hoping to get some direction from this group. ***Mary Ellen Pease mary.ellen.pease@gmail.com***

We had an interesting talk by Daniel Fulton from the University of Birmingham, UK (https://www.birmingham.ac.uk/staff/profiles/inflammation-ageing/fulton-daniel.aspx) where he spoke about long-term live imaging of oligodendrocyte myelination in organotypic brain slice cultures. Maybe some of his techniques might be useful? His talk is at the end of the session located at this link: https://www.youtube.com/watch?v=0L7tDQ2vDsM Phillipa Timmins phillipa.timmins@aurox.co.uk

Staining of organoids within matrigel is a challenge and results in bad signal-to-noise ratios, but if the organoids are expressing GFP, or even better long wavelength fluorophores, imaging can be performed within the matrigel matrix. A dilution of matrigel with medium works well and dilutions of up to 1:5 are applicable. To increase the number of organoids, close to the coverslip, you can place the dish on an ice pack while you seed the matrigel-medium-organoid suspensions and keep it on ice for 5 minutes. Within cold matrigel the organoids sink by gravity and you'll have more organoids within the working distance of the lens. I recommend spinning disc microscopy, due to low photo-toxic stress. Matrigel and 2-photon imaging might be challenging due to the second harmonics of the collagen matrix. Philipp Tripal philipp.tripal@fau.de

Some time ago I was helping a user with their zebrafish embryos. The system was an LSM 780 upright with dipping lenses. The embryos were maintained in position by pressing small dimples into the top surface of (I think) agarose, then resting each embryo in a dimple, covered in water. It was sufficient in most cases to prevent drift. Michael Doubé mdoube@cityu.edu.hk

Have a look at CyGel. You can get it from Biostatus and Abcam also distributes it in the US. It's a liquid at 4°C and it gels around 22-27°C. It is reversible once you lower the temperature. We have not tried it with 2-photon imaging or organoids, but it worked quite well in our hands with cells and fluorescence microscopy. Zbigniew Mikulski mikulski@lji.org

We kept organoids alive and apparently happy for 12–18 hours on a microscope stage using embedding in Matrigel. In addition to organoid imaging alone, cells can be added to Matrigel to watch how they interact with organoids. Imaging in the paper below was at low mag, but (not used in the paper below) I fixed and labeled the organoids after overnight imaging to see the same ones at high resolution with confocal, so confident this would have worked live at high res too. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5716041/ Michael Cammer michael.cammer@nyulangone.org

We regularly image organoids embedded in matrigel on an LSM710 (invert) for up to 4 days. We've imaged GFP, Venus, mBanana, RFP and mCherry, using the standard single photon laser lines. The organoids have been embedded in a matrigel cylinder around 3 mm in diameter x

5 mm deep, attached to the bottom of a 24-well plate with media filling the well. This has kept multiple organoids per matrigel cylinder viable for the full timecourse. Running non-imaged controls alongside has not shown any noticeable phototoxicity. We've been taking Z stacks up to approximately 1.5 mm from the plate bottom without any problems or need to increase gain or laser power through the stack, so we're not finding the matrigel to be a problem. The caveat here is that we've been able to get away with using a 10x/0.45 objective for our needs, but if you have the working distance in the objective you need, it should be worth a try. Richard Lisle richard.lisle@ludwig.ox.ac.uk

Glycerol/Type G Immersion Liquid

Confocal Listserver

Does anyone know if glycerol immersion liquid is in fact merely glycerol, and if off-the-shelf (molecular biology grade) glycerol can be used instead of designated glycerol immersion liquid? If so, can the refractive index be lowered simply by adding water and thoroughly vortexing/sonicating to get immersion liquid similar to Type G? ***Jan Tonnesen janton@gmail.com***

I have in the past used various solutions as immersion liquids for my glycerol immersion lens. You can straight up use glycerol, but with a refractive index of n=1.47 you ideally want to bring it down (with water) to n=1.45 to match the specifications of the lens. You can correct for some mismatch using the correction collar, but this takes quite some time and practice, so best keep it to a minimum, if possible. This works perfectly fine, with two main caveats. First, over time the water in the mixture evaporates, causing the refractive index to tick up slowly. This induces increasing aberrations, or requires frequent AO, correction collar readjustment, or reapplication of fresh immersion liquid - all of which you would like to avoid when performing live imaging. If I am correct, branded immersion liquids (e.g., Leica Type G) include additives aimed at slowing the evaporation of water. This would slow the process of RI change, requiring less frequent realignment. Also, this means the refractive index of the "stock" immersion liquid is more constant and predictable, leading to less time spent on pre-aligning the correction collar/AO at the beginning of an experiment. Especially if the vial of pre-mixed immersion liquid sits on a shelf for long periods of time in between imaging. That being said, I have imaged with both, and you can easily make both work. Especially if the RI is tweaked a little to pre-compensate aberrations (such as are incurred by imaging deeper within living tissue), this is best done by either pre-mixing the immersion liquids, or by simply buying RI-matched liquids with the appropriate RI. I have used Cargille immersion liquids in a range of RIs to do this and they worked fine. They are slightly pungent, however, and can be more unpleasant to work with. Nicolai Urban nicolai.urban@mpfi.org

We have used glycerol objectives for many years. The advice from Leica has always been to use glycerol/water 80:20. Konstantin microscopia-ibis@us.es

Phase Contrast Microscopy

Microscopy and Confocal Listservers

I thought I understood how phase contrast microscopy works, but then I was reading MicroscopyU (https://www.microscopyu.com/techniques/phase-contrast/introduction-to-phase-contrast-microscopy) and other sites and now I am confused. My understanding is that it works because of refractive index differences in different parts of the light path (cells) leading to retardation of refracted light and eventual phase differences relative to un-refracted (surround) light that passes through

the thinned area of the phase plate in the objective. However, in the web site indicated, and others, they use refracted light and diffracted light almost interchangeably in explaining phase contrast. To my "biologist" level of understanding, diffraction and refraction are very different phenomena and I did not think that diffraction changed the optical path length like refractive index differences. Refraction makes total sense to me in the context of phase contrast, but I don't see how diffraction is relevant. Can someone explain what I am missing? Thanks, ***Dave Knecht david.knecht@uconn.edu***

I am also a biologist and sometimes have a hard time understanding such complex physical phenomena. My understanding is:

- Diffraction is due to the interaction of the focused incoming light on the sample. Diffraction is the reason why an object is visible in light and electron microscopy.
- Refraction is related to the change in light speed through a change in the medium (lens) thickness, and the phase ring (which you must choose according to the objective used).

The principle here is to separate in physical space the normal (not diffracted) light from the light diffracted by the sample. If you look at the picture shown in the link you gave, you see only a yellow beam before the sample but after the sample you see a light pink color between (and also outside) the yellow beams (called direct surround light). This is the diffracted light (the lines point to the rosy color between the yellow light beams). I think I identified your problem: the phase difference doesn't occur by passing through the sample, it occurs due to the phase ring placed after (or within) the objective lens. The interaction of light with the sample gives diffracted, not refracted light (or perhaps some negligible refraction). The diffracted light (by the specimen) will be refracted by the objective lens just as the non-diffracted light is BUT it will not strike the lens at the same place. Only the non-diffracted light will pass through the phase ring (because the ring is calculated to be in the path of non-diffracted light only) and this will result in a phase difference between the light diffracted by the specimen and the light that passes through it unchanged. This difference is then reconstructed on the image plane to give contrast. Stephane Nizets nizets2@yahoo.com

With due disclaimer that I am doing ion and electron beam processing and my recollection of light optics is from loooong time ago – diffraction encompasses a multitude of phenomena, including separating spectral components of white light and possible interference between waves diffracted at different locations of the object. Separation of the spectrum with white light illumination and interference could both produce a delayed wavefront resulting in phase contrast. My feeling however is that most of phase contrast from thin, uniform, transparent biological specimens would be produced by differences in the index of refraction. The "Diffracted light" notation on the Microscopyu page refers to separation of diffraction orders within the light path of the microscope. During diffraction contrast, imaging direct light (DC background) is blocked by the illumination aperture (condenser annulus) and the diffraction plate, while first-order diffracted light is passed to the image plane to form phase-contrast images. I'm sure that many people with more current and fundamental knowledge of optical microscopy will correct and expand my qualitative and exceedingly hand-waving explanation. Valery Ray vray@partbeamsystech.com

Perhaps, the confusing part here is that we usually consider that diffraction occurs at sharp boundaries between transparent and opaque. But diffraction can also be caused by boundaries between parts with different refractive indices. So, phase contrast utilizes diffraction which is caused by refraction. Such is at least my understanding. Mike Model mmodel@kent.edu

I think that your understanding of phase contrast is correct and that you have explained it yourself properly and in a concise way. Diffraction and refraction are different indeed. It is in fact the diffraction of light that plays an important role in phase contrast microscopy. Have a look at the Leica tutorial: https://www.leica-microsystems.com/science-lab/the-principles-of-phase-contrast and figure 4 in the link you shared:

1. The illumination light passes through the annular ring in the condenser, resulting in a hollow cone of illumination.
   - 2.1 A higher refractive index in the sample causes retardation, on average generating a phase shift of -1/4 λ in the light that interacted with the specimen compared to freely passing light.
   - 2.2 Light interacting with the specimen (cell, granule, nucleus…) is diffracted to the outside of the illuminating light cone. The smaller the object, the larger the angle of diffraction.
   - 2.3 Light that does not interact with the specimen is not diffracted and hence stays on the inside of the illumination cone.
3. In the phase plate, only the light on the inside of the light-cone is then advanced +1/4 λ.
4. This results in a phase difference between illuminating light and sample light of 1/2 λ, generating destructive interference and hence maximum contrast in the imaging plane wherever there is a structure in the sample. Here is also a very nice iBiology talk on the subject: https://www.ibiology.org/talks/phase-contrast-microscopy. Kai Schleicher kai.schleicher@unibas.ch

I've found the best way to understand phase contrast is on a phase contrast microscope. On a phase contrast microscope, take out one of the oculars so you can see the focal planes. In the path without the ocular, you should see a ring of light perfectly aligned to an annular neutral density filter (switch between brightfield and phase contrast to see how the illumination perfectly aligns with the ND filter). If they are not aligned, then the annulus is not set up properly and you are not getting phase contrast. Next make a slide with a Kim-Wipe or lens paper on it or grab an unstained test slide (although tissue paper makes this effect very clear). As you move the sample into the light path, you will see all the diffracted light miss the annular ring of light and fill the rest of the focal plane. Move the sample out of the light path, and you will see this diffracted light disappear. Next, get a poor phase object like adherent cells or anything else that is hard to see with brightfield, and put it under the phase contrast microscope. Once you are focused on the sample, look at the focal plane (side without the ocular) and slide the annulus out of the way just a bit (it should look like the sun just peeking around the moon after a solar eclipse). Now look at your sample, and it should look like just brightfield (i.e., poorly contrasting). Then put the annulus back into place, while looking at the sample, and you will see a sudden jump in contrast. This is one of the striking features of phase contrast where it really is all or none, and if the annulus is at all out of alignment you just get brightfield.

As others have said, phase contrast is fixing a fundamental issue with brightfield imaging of poorly diffractive objects (such as adherent cells). The issue is that brightfield microscopy works by having the diffracted light undergo a phase shift relative to the undiffracted light (as it follows a different path length in the microscope). Then, these rays are allowed to interfere with each other at the image plane, producing an image. You can see this effect in brightfield in a similar manner to

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the phase contrast trick described above, but instead close the aperture stop and you will again be able to see the diffracted light separated from the non-diffracted light in the focal plane. The problem with poorly diffracting objects is two-fold, 1) very little light gets diffracted leaving little light for interference, and 2) the phase shift of the diffracted light is very small. The end result being that samples like adherent cells only cause a very small decrease in intensity, and since we perceive light on a logarithmic scale, this is very hard for us to see. Therefore, phase contrast fixes both of these issues. One way (which you can see by looking at the focal planes) is that it uses a cone of light as the illumination pattern (due to the annulus at the front focal plane), and this cone perfectly lines up with an annular neutral density filter (the phase plate) at the back focal plane. This ring illumination is a clever way to spatially separate the diffracted light from the undiffracted light at the focal plane, allowing us to attenuate the undiffracted light without impacting the diffracted light, balancing the amount of diffracted and undiffracted light. Why use a ring to do this instead of, say, an aperture stop? The ring illumination simply preserves more of the illumination NA and therefore preserves more resolution.

However, the phase plate doesn't stop there, as its name suggests, it also enhances the phase difference between the diffracted and undiffracted light (ideally such that the highest diffractive orders are phase shifted to 180°) such that the sharpest edges get perfect destructive interference. Now at this point, you may be asking yourself, how do they know how much to attenuate the undiffracted light (i.e., how dark to make the annular ND filter) and how much of a phase shift to impose, as some samples may be more diffractive than others. And this is why there are actually many different phase contrast objectives with different phase plates. In life sciences, most of the time we're only using phase contrast to check cell culture, so we wind up dealing with just one phase plate optimized for that task. Hope this helps, and I really do recommend checking it out on your own microscope. I've found with students, looking at the correlation between the focal plane and image plane with and without a sample can really inspire that "aha" moment. Ben Smith benjamin.smith@berkeley.edu

SEM Filament Vibration

Microscopy Listserver

Looking for feedback from the EM community. It was recently suggested to me that what appears to be wave-like image distortion normally attributed to electromagnetic interference (EMI) could actually be induced by vibration of the tungsten filament that results from incorrect centering or height adjustment in relation to the Wehnelt. Has anyone looked into this or know if this is possible? ***Mike Toalson miketoalson@gmail.com***

EMI effects can be easily checked when you go to a low working distance like 6 mm and set SYNC on scan at 50 or 60 Hz (whatever your power grid has). If effect decreases you have some EMI influence. Using a magnetic field cancellation system like a Spicer 22 will greatly help. Only if this is ruled out then it might be something as strange as filament vibration. Is it dependent on HV value? It might also be a problem with the scan amp or a power supply there. Stefan Diller diller@stefan-diller.com

Noise is the usual subject for bickering between users complaining about noise and equipment manufacturers unwilling or unable to resolve issues with an instrument, or convincingly demonstrate that the problem originates from the environment. Fortunately, there are a few tests you can run on the SEM to point in the direction of the root cause:

1. As already suggested, change working distance. If the root cause is EMI picked up by the electron beam, then a larger WD will increase the noise. The same will happen if the root cause is mechanical vibration of the source. But if the source of the noise is in mechanical vibrations picked up by the stage or body of the instrument, then the magnitude of the vibrations wouldn't depend on working distance.
2. Change acceleration voltage by a lot, like 3kV to 30kV (if you can). If the source of noise is EMI picked up by the beam, then lower-energy electrons would be more susceptible to it, and the magnitude of the noise with the lower acceleration voltage would increase.
3. Go through the range of acceleration voltages. If the source of the noise is some kind of resonance or cyclic process taking place with the source, or oscillations in the high-voltage power supply, then you may see not only magnitude, but also the frequency of the noise change.
4. Tap gently on the SEM column with a screwdriver. You will see additional noise but look instead at how such tapping affects the original noise that was present. If tapping enhances the original noise, or maybe suppresses it for some time, then the likely cause is some kind of mechanical resonance in the column or with the source.
5. Get a stethoscope (or surface-pickup microphone) and listen to sounds of the column. If you hear something when the source is up, and disappearing when the source is off, then there is a possibility of the root cause being associated with the source operation (oscillations, resonance, etc.).
6. Connect the surface-pickup microphone to an audio input of a laptop and run the audio spectrum analyzer - frequency and changes may point to the root cause. For example, if frequency matches rotation of the turbo then that could be the original source of the noise.
7. Connect the spectrum analyzer to the imaging output of the SEM. There will be frequencies associated with line scanning but look for something related to 50/60Hz or 100/120Hz. This is indicative of poor filtration or a ground loop somewhere. Do this with the electron beam blanked, open, and scanning. Differences could pinpoint which part of the SEM circuitry is affected.
8. If a spectrum analyzer is way too exotic an animal, then try working out frequency of the noise from the periodicity of waves and how it changes with change of the scanning frequency.
9. Switching the frequency of the high-voltage power supply or intermodulation between switching frequencies of its various modules could be the culprit, if decoupling or filtration is inadequate.
10. Change imaging modes of the SEM. If noise is present in one of the modes and not in another, the root cause is in SEM electronics.
11. If everything else fails, get engineering help from someone with experience in noise troubleshooting. Valery V. Ray vray@partbeamsystech.com

Loading Magnetic Nanoparticles into a TEM

Microscopy Listserver

I have a user that wants to do HRTEM on magnetite nanoparticles. My concern is that the nanoparticles will be pulled away from the carbon-coated grid by the strong magnetic field of the TEM lens and contaminate the column. If anyone has a similar experience and can provide advice it will be much appreciated. The sample is powder which I usually dilute with alcohol, then apply a drop of the suspension onto a carbon coated grid. ***Fei Long fei.long@queensu.ca***

You can use another carbon-coated grid on top of the grid loaded with magnetic nanoparticles. Ravi Thakkar ravi.thakkar369@gmail.com

The EMF is here if you have a magnetic field and movement. So insert the sample without the magnetic field and then use the

microscope with a very slow movement. For retracting the sample also use no magnetic field. Michel Ribardière m.ribardiere@jeol.fr

This is indeed NOT a good idea. The particles WILL be captured by the lenses. I am afraid the user needs to find another method. An alternative may be to embed them and analyze sections? By finding the right dilution, one should be able to image only 1 isolated particle. Stephane Nizet nizets2@yahoo.com

There seems to be some significant misunderstanding of the scale of forces applied by the 'strong magnetic field' inside a TEM. I've been looking at magnetic nanoparticles of various kinds in every type of TEM/STEM system for many years and never had any problems. The van der Waals forces - the electrostatic forces sticking your particles to a grid are VASTLY stronger (several orders of magnitude) than the magnetic moment for micro- or nanoparticles inside a TEM. As long as you are not dumping vast amounts of this material onto your grid (a good rule of thumb is if you can see anything by eye in the suspension or on the grid after deposition) you are generally safe. A good approach to be really sure is to pass a rare earth magnet over the sample after deposition to pick up any larger boulders/chunks (thanks, Nestor). The real risk for magnetic materials in TEMs is where a bulk specimen can become magnetized as a single or set of aligned domains (such as a 3mm chunk of ferritic steel), then the combined magnetic moment can tear a sample from a spring clip. A safe holder to use in these cases is always a screwed hexnut. Matthew Weland matthew.weyland@monash.edu

For a properly loaded TEM sample, the risk of nanoparticles being ripped from the support is very low. By properly loaded, I mean that a small amount of particles are evenly distributed across a TEM support grid. You want to avoid clumps that are visible in a stereoscope or microscope. Large clumps can be weakly adhered, or not adhered at all, and could be pulled to one of the pole pieces. Your plan to dilute with a solvent and drop onto a TEM grid is perfectly reasonable. Just check it in an optical microscope to make sure you don't have any clumps. If you do, dilute and repeat on a new grid. I've looked at countless magnetite (and other magnetic NPs) and have never observed any particles being removed from the support film. On the TEMs I've used to image these samples, I've inspected the objective pole pieces carefully during service and they were clean, at least at the level of observation permitted by an optical microscope. You can monitor the objective stigmator values over time to see if you're getting gross contamination, but it would take a *very* large number of NPs/clumps to make a perceivable impact. Now, bulk magnetic samples are a very different story! I can tell you stories about "hairy" pole pieces and magnetic samples making their home inside a pole piece (I'm guilty of the latter, I'm afraid to admit). Chris Winkler crwinkler@ncsu.edu

I have done what Ravi suggests and it works fine. Just takes a little time to align the grid bars of the two overlapping grids. Roy Geiss roy.geiss@colostate.edu

Contrast Enhancement Strategies in TEM

Microscopy Listserver

We've recently moved to non-radioactive staining agents and are finding that these alternatives generally offer less contrast than their radioactive counterparts. In an effort to improve imaging of these lower contrast materials, I've tried to think of every major way to increase contrast from the TEM side of things. Our TEMs are materials 'scopes and we don't have access to a dedicated bio TEM, so I'm working with what I have.

I've moved to lower voltages, from 200 to 80kV, used as small of an objective aperture as I can get away with, applied a generous amount of defocus during imaging, and used the GIF to form images from the elastic contribution. Am I missing anything major besides digital manipulation of brightness, contrast, and gamma on the images? Is it worth spending the time and effort to align at 20kV to improve contrast in thin sections? Thanks for any advice you can share. ***Chris Winkler crwinkler@ncsu.edu***

I'm not sure what you mean by "GIF" in this case (I'm assuming you don't mean the file format), but if this isn't an aperture … since you have materials 'scopes, you have a diffraction/field of view aperture. You can use this aperture to increase the contrast without any resolution loss (since this aperture isn't in the objective lens). It will give you a bit of resolution improvement by removing peripheral electrons, but the main negative effect is the vignetting. On our TEM, we can't use even the largest (200 μm) below ~10kX. The contrast increase is less than that from using the objective aperture, but it's noticeable. Phil Oshel oshel1pe@cmich.edu

I guess I don't understand how the SA aperture increases contrast since it is in an image plane not a diffraction plane. You are referring to the SA aperture, right? As a note… GIF is "Gatan Imaging Filter". Chris is trying to improve contrast by energy-filtering. Hendrik O. Colijn colijn.1@osu.edu

I have the same question as Henk. Are you referring to the aperture that is located at the intermediate crossover in JEOL microscopes? I do miss that aperture because you could use it as a pseudo objective to increase contrast in TEM mode, improve BF contrast in STEM mode, and most usefully employ it during EDS. Or do you mean using the selected area in low mag to improve contrast? As Henk said, the GIF is the Gatan Energy Filter.

I mean the selected-area (SA) aka diffraction aka Hitachi's FOV (field-of-view) aperture. It increases contrast just because it blocks strays and peripheral electrons (like the fixed apertures do) and helps cut aberration a bit by blocking peripheral electrons. Just a side effect of being an aperture. Doesn't matter if it's in an image plane or a diffraction plane. Not a low mag aperture. (I'd have to look at a column schematic to know if this is where the intermediate crossover is in a JEOL. It's below the objective lens, at the first intermediate in the Hitachi 7700.) The effect isn't as strong as with an aperture specifically placed to increase contrast (in the objective lens), but it's still there. Phil Oshel oshel1pe@cmich.edu

OSU also only has an analytical TEM and it is difficult to image biologicals. Lucky for me I can do STEM imaging on one of our SEMs. With a larger field of view and the extra staining I do it works out great. I was introduced to O-T-O-T-O staining a few years back and now I use it all the time. You might not need to do all three osmium stainings but this method has eliminated the UA or alternative from the protocol. I have attached the protocol, but I have modified it some over the years. I cut the osmium staining time in half. I cannot stress enough how much easier my life is with this staining and no more post-microtome staining. Good luck. Teresa Sawyer sawyerte@science.oregonstate.edu

Thank you for the paper and the protocol. I'll pass it along to the scientist in charge of sample prep. The idea about switching to STEM in the SEM is quite interesting. Thank you! That's definitely something we will test out. Chris Winkler crwinkler@ncsu.edu

STEM is definitely the next item on the menu. The trick about inverting the HAADF image is a good one if you don't have a BF detector! I'm all too familiar with samples blowing up under the beam. A thin coating of carbon on the exit surface (or sometimes both sides, in my experience) usually solves it as you indicate. If only the carbon coater was in the same building as the TEM. Thanks for the advice, Chris Winkler crwinkler@ncsu.edu

Hi Henk, I'm not sure about the kind of microscope Phil was referring to, but as for FEI microscopes such as the Tecnai the diffraction plane is at the selected aperture plane only when in low mag mode (objective lens off). I guess in Phil's TEM it might be something similar. Chris, I have used a JEOL TEM a few times, so I am not familiar with it. But yes, there might be a way of switching off the objective in low magnification mode. You will get a better contrast. Another possibility that might work, as pointed out by Henk, is to use the ADF or HAADF detector to do dark field imaging and invert the image contrast. I've done (inverted contrast) ADF imaging on non-stained tissues and it works (with that sample I could see absolutely nothing in TEM mode). Another thing you can do is BSE-SEM imaging of the sectioned embedded block's surface. Of course, you need to carbon coat its surface before. Use the right SEM settings to reduce the interaction volume. Maybe you can try lower kV, lower WD. Good luck. Erico Freitas ericotadeu@ufmg.br

I have a Hitachi 7700. The FOV (field-of-view), aka selected area aperture, works at all mags, not just in low mag. I suspect the same is true for any TEM with a SA (selected area - let's expand the abbreviations in the emails). Just do your imaging as normal, and if you need a contrast bump without losing resolution, put in the SA/FOV aperture. It's just adding another aperture, not changing any imaging parameters or any electronics. You do lose field of view at mags <10,000 or so, depending on the aperture size, but that's it. Assuming you have a manually variable aperture and not some computer-controlled thing that only works when and how the computer thinks it should. If that doesn't exist, it's coming. Phil Oshel oshel1pe@cmich.edu

Hi Chris, what TEM do you have? If it is a FEI/ThermoFisher you could try imaging the samples in low magnification (LM) mode. In LM the objective lenses are off (you will see the strength value around 6%), and in that case the diffraction lenses act as the objective lens (you may even want to use the selected area aperture). In FEI machines you will have better contrast in LM mode. I know the magnification is not that high in LM mode, but you may still benefit from using the GIF camera that will give extra magnification. Best wishes. Erico Freitas ericotadeu@ufmg.br

I can confirm BSE-SEM imaging can really be advantageous even on ultrathin sections. A thin carbon coating is a must, as Erico mentioned in his message. In BSE imaging mode one can play with accelerating voltage, concentric back-scattered detector rings and beam deceleration settings. With an optimal setting, you can image a really huge section on a TEM grid with almost no interference of TEM grid bars. Some years ago we used such approaches for imaging of whole ultrathin sections through mice teeth. We were searching for places with Tomes' processes in ameloblasts and we were successful. Oldřich Benada benada@biomed.cas.cz

Thank you to all for your responses and advice! I want to summarize the responses I received regarding my query on how to improve contrast on low contrast samples in a materials science TEM:

1) Buy a Bio TEM with a large gap objective optimized for high contrast.
2) Adjust staining protocols--check pH levels, switch to radioactive stains (EH&S now makes this difficult in some universities), supplement non-radioactive stains with lead citrate, tannic acid, or other agents, and so forth. One responder linked me to the following protocol that worked wonders for her: https://www.sciencedirect.com/science/article/pii/S1047847714002378
3) Try to minimize contribution from the grid by moving to ultrathin carbon or plasma cleaning thicker carbon to thin it down.
4) Acquire EFTEM SI and generate jump-ratio maps around the plasmons peak, carbon peaks, etc.
5) Switch to STEM and see if contrast is improved when using ADF or BF detectors. If you only have an ADF detector, invert the image contrast to give a pseudo BF image (more acceptable to people used to TEM). Similarly, switch to the SEM and try STEM or BSE imaging at 30kV.
6) Send a sample to Delong Instruments and see how it looks at 5, 15, and/or 25kV. Also, try working at lower voltages like 40 or 60kV.
7) Work in low mag (LM) and use the selected area aperture to further improve contrast. LM contrast will be inherently higher as the objective lens is turned nearly off.
8) Sum/stack several images to improve SNR. For thin sections with little contrast and no outstanding features for correlation, this will be tricky.
9) Replace one of the objective apertures with a phase plate.
10) For JEOL and Hitachi microscopes, use the field limiting aperture located below the objective to improve contrast.

Thanks again to everyone for all the help. We'll be trying STEM in the TEM and SEM next. Chris Winkler crwinkler@ncsu.edu

MT

Crossword Puzzle Answers

See puzzle on page 60.

1 C	2 O	3 A	4 U	5 T	6 H	7 O	8 R	■	9 A	10 M	11 M	12 I	13 N	14 O
15 A	N	N	O	T	A	T	E	■	16 E	D	I	T	O	R
17 M	T	■	18 T	A	N	■	19 V	20 S	■	■	21 C	I	N	A
22 B	O	23 O	■	24 B	G	■	■	25 J	O	U	R	N	A	L
R	■	26 A	27 L	L	S	28 T	29 A	R	■	■	O	■	■	■
30 I	31 S	S	U	E	■	32 A	C	■	33 D	R	A	34 F	35 T	36 S
37 D	O	I	■	■	■	38 G	A	39 L	A	■	40 N	O	O	N
41 G	O	S	L	42 O	43 W	■	44 I	F	■	45 C	A	R	P	I
46 E	N	■	■	47 G	A	48 L	■	■	■	49 I	L	K	■	P
■	■	50 M	51 I	C	R	O	52 S	53 C	54 O	P	Y	■	■	■
55 B	56 S	■	N	■	■	57 G	U	H	R	■	58 S	A	60 T	61 O
62 E	T	■	63 I	64 M	65 D	O	N	E	■	66 G	I	V	I	N
67 S	E	68 C	T	I	O	N	■	69 W	70 E	B	S	I	T	E
71 R	E	V	I	E	W	■	72 T	O	N	Y	■	73 E	L	L
74 A	R	S	O	N	■	75 R	A	N	G	E	■	76 W	E	B