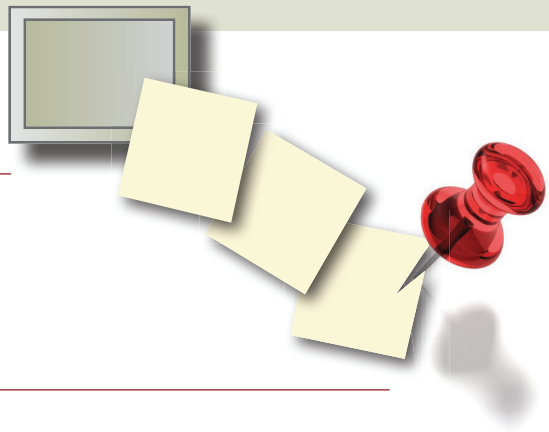
NetNotes



Edited by Bob Price
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Everhart-Thornley Detector and Auger Electrons

Microscopy Listserver

I was wondering if an Everhart-Thornley detector also measures Auger electrons from the sample? I don't see a reason why it should not. Can somebody shine some light on my miserable ignorance? Best regards, ***Stephane Nizet nizets2@yahoo.com***

If the low energy electrons make it to the detector, they would register as noise. Without the right detector these energies are too low to separate for any useful purpose. Plus, at those vacuum levels of an SEM system the adsorbed gases (contamination) would dominate the Auger electron emission. Kimball Skinner kls6_30@yahoo.com

Detect them, yes; analyze them, not so much. How would you measure their abundance and energy? The ETD detector was not meant for spectroscopy. I suppose another problem would be differentiating the Auger electrons from other low energy electrons. Warren Straszheim wesaia@iastate.edu

You would need a retarding field analyzer (RFA), cylindrical mirror analyzer (CMA), or similar setup. Then a ramp generator to scan the analyzing voltage. Next a function generator and lock-in amplifier to differentiate the signal and to isolate the tiny Auger signal. And yes, the 50eV to 1000eV (0.05 to 1KeV) Auger signal would be dominated by the carbon and oxygen atom adsorbate on the surface. Jim Quinn james.quinn@stonybrook.edu

Consider that the ET detector is made of 2 elements: a polarized front grid and a photoluminescent screen to convert the energy of the detected electron into light. To increase the signal intensity, the screen is positively polarized (≈ +10keV) to post-accelerate the electrons once they cross the grid, and a photomultiplier (or any suitable type of light detector) behind the screen to convert light to an electric signal. The front grid is polarized to choose which of the emitted electrons will be allowed to reach the luminescent screen: a positive grid polarization (≈ +200…+300 V) will let all emitted electrons to reach the screen. However, the electric field created between the grid and the sample will attract most of the (low-energy) secondary electrons (SE) toward the grid regardless of their emission direction, while backscattered electrons (BSE) (higher energy) will be less sensitive to the field and not affected by this "pumping" effect. This is the SE contrast mode, even if some BSE and Auger are also detected. A negative potential (≈ -200…-300 V) will repel most of the low-energy SE electrons and let only BSE + Auger + some high energy SE electrons enter the detector if they were emitted toward the detector (a quite low solid angle) and with an energy larger than 200…300eV. This is the back-scattered contrast mode. The absence of "pumping" leads to a much lower signal/sensitivity. Conclusion: the Everhart-Thornley detects Auger electrons, but not only them. The relative proportion of SE, BSE and Auger electrons reaching the detector depends on the "pumping" effect due to the grid polarization at low energy (that is, on the selectivity). Therefore, there is a world between "detect" and "measure". A more detailed answer would require knowledge of the energy range of the Auger electrons being considered. Philippe Buffat philippe.buffat@epfl.ch

Question about the Resolution Limit of Field Emission-Scanning Electron Microscopes (FE-SEM)

Microscopy Listserver

Although I have some experience in analytical SEM, I am completely new to the field of FE-SEM and I am not really aware of the true limits of the system. I inserted a gold-on-carbon specimen into the chamber with high vacuum, brought the specimen as near to the pole piece as I could, used the smallest probe current setting and aperture, and imaged using the in-lens detector. I worked at 1–5kV to limit penetration of the beam into the specimen. Using a magnification of 300,000X, I did my best to correct the astigmatism but could not get a sharp image. Is there something I still need to consider, or did I simply reach the limits of the system? Many thanks in advance. ***Stephane Nizet nizets2@yahoo.com***

It depends on the FE-SEM you have. When I used a Hitachi S4700 at 1 kV, the best we could get was around 80,000X. On a Hitachi S4800, at 1kV, we get 800,000X (1nm). On the Hitachi SU9000 the resolution is at least 0.2nm. It sounds like you need a bake-out and perform a series of bombardment sessions to get a better picture. Elaine Humphrey ech@uvic.ca

I would suggest you contact the applications team from your microscope manufacturer so you can understand the limits and best methods to tune the system for optimal imaging. They will be able to provide this advice in general, but it also sounds like you could benefit from a training session. Most of the manufacturers also offer live remote training options. Kimball Skinner kls6_30@yahoo.com

I don't know what platform you are on: Hitachi, JEOL, TESCAN, or ThermoFisher, but all should give crisp images at 300,000x. With a field emission gun source, all manufacturers should be able to approach 1.0 nm resolution at 5 keV with the in-lens detector. The 300,000X relative mag should be easily achievable with good crisp images on a FE-SEM. Have you performed a lens alignment to make sure there is nothing amiss? In a ThermoFisher instrument, a lens alignment can show a host of issues including a bad lens supply and control boards that have gone bad. There is the lens bottom test where charging sphere is placed in the SEM and imaged at low mag. An image of the pole piece should be seen and appear

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normal. FEG sources are generally good for 2–3 years of continuous service or up to 10,000 hours. I do know that with time the FEG source can get a funny shape to it. Pete Eschbach peter.eschbach@oregonstate.edu

Two ideas: 1. The filament tip might be damaged. Check the resolution at 30 keV; if it is OK, then the tip is most probably okay, and you should fix the low kV alignment. 2. Depending on your instrument, the best resolution might be obtained at a higher probe current than the minimum setting. Tomáš Hrnčíř tomas.hrncir@tescan.com

In addition to the gold standard used to measure the resolution, I used real samples such as metallic PVD nanolayers. Three factors need to be improved to get a 1,000,000X image: 1. The magnetic interference of the Faraday cage coil system should be minimized; 2. The anti-vibration shoe system should be minimized to remove any floor chattering effect; 3. The plasma cleaning system should be used to eliminate carbohydrate contamination. Antonio Carlos Joaquim acjoaquim@gmail.com

Along with all the excellent input and advice already posted, I'd also recommend cleaning the Au-on-C standard too. The surface will inevitably become contaminated over time, adding to surface contamination and resolution problems during high-resolution imaging. Plasma or ozone cleaning both work well, plus try storing the standard in a clean cabinet, even under vacuum. Chris Jones c.jones@nhm.ac.uk

Use of a TEM Wobbler for Perfect Focus

Microscopy Listserver

I'd like to draw on the collective wisdom of the microscopy community one more time. I have a Philips CM12 TEM and I have a disagreement about the wobbler function. The premise is, if the optics and column are perfectly aligned, even if the sample is out of focus, the wobbler will not split the image into two and allow you to focus. In the twenty years I've hung around TEMs, I've never seen a scope that is so perfectly aligned, so in tune with the universe, so one with itself, that the wobbler doesn't wobble a defocused image. ***Frank Karl frank_karl@ardl.com***

I've been involved with EM since 1974 and I have never heard that. The wobbler is a focus aid. I think this is one of those EM myths. Tom Bargar tbargar@unmc.edu

The manual for the CM12 and CM10 we used to have stated the wobbler was for focusing at 25,000X and below. Only. It was useless above 25,000X. But it didn't matter if the column was perfectly aligned, it would still wobble the image if it were out of focus. There was a separate wobble function for alignments. (If I remember right - we got rid of both the 12 and 10 years ago, and with all the microscopy chemicals, concussions, and admin wobblers, my brain may be too wobbled to remember.) Phil Oshel oshel1pe@cmich.edu

This may be a hardware problem like misalignment of coil voltages. Does this problem exist at all magnification steps? I suppose you use a digital camera on the TEM. Why not use the Fourier window (which most camera software has) to do all your corrections more easily? Stefan Diller diller@stefan-diller.com

It would be best to define what you are "wobbling." All of the following are possible and all have effects on alignment and the image: HT, gun lenses, monochromators, condenser lenses, objective lenses, projector lenses (not done often), beam deflector/tilt coils, image deflector/tilt coils, all stigmator coils. I've been known to wobble all of them to align my instrument. Nestor Zaluzec anl.nestor.zaluzec@gmail.com

I'd be interested to know which (OL or HT wobblers) are best to get closer to coma-free alignment on JEOL. Another trick for the image wobbler is astigmatism correction (at least on JEOL instruments): 1. focus with WBL-X and adjust O-ST for minimum movement. 2. set the OL to halfway between minimum movement of WBL-X and WBL-Y. 3. correct O-ST for no movement. 4. check Thon rings for confirmation or touch-up. Mike Marko mike.marko.em@gmail.com

I started a conversation about perfect alignment and the wobbler for focusing the image. That's been resolved and I thought I'd share the result. I want to thank everyone who contributed to that conversation. In the dark while using the wobbler, someone reached over and while using the multifunction knobs accidently changed the angular displacement to zero. It could have been me; it could have been the new guy; it could have been a service technician. It's a small group of suspects. Frank Karl frank_karl@ardl.com

Problem Getting Top Off Pin-Type Autogrid Grid Boxes

3D EM Listserver

We have been having a problem where users tighten the tops of autogrid grid boxes and the tool's metal tip unscrews before the top does. Has anyone found a way to better secure the metal tip of the grid box top tool so it won't spin, or to ensure the grid box top will loosen before the tip of the tool? Thank you. ***Robert Grassucci rg2502@cumc.columbia.edu***

We use a Pella autogrid box tool for overtightened boxes. These tend to frost up quickly, but since there is no screw, it does not come apart. MiTeGen also makes something similar. Richard Walsh richard_walsh@hms.harvard.edu

Applying a bit more downward force than you'd first feel comfortable with seems to tighten up the threads in the tool so they don't slip. I've never broken the pin or anything else, but it's not inconceivable. Just push straight down. You can also swap out the official tool for a mechanical pencil (Staedler Mars technico 788), with which it's hard to overtighten in the first place. Michael Elbaum michael.elbaum@weizmann.ac.il

I have tried that, and the tip comes loose again. I think I will try crazy glue or epoxy as suggested by Bill Rice. What might be a better design would be to reverse the threads so loosening the top would tighten the tip and tightening the top would loosen it. That way it could not get tightened. Robert Grassucci rg2502@cumc.columbia.edu

Developing better tools for handling grid boxes is an area that we have been looking at recently. As mentioned, many labs are using the "off-label" mechanical/drafting pencil approach. These pencils are far from optimal for gripping or for use in LN_2. We would welcome input from the community on what they might like to see in an optimized gripper tool, or other tools, in the sample prep workflow. Benjamin Apker benjamin.apker@mitegen.com

We had a go at modifying the lids with two small blind holes to fit a tool with matching pins. It worked very nicely, but eventually we gave up due to the hassle and the tendency of the modified lids to get lost. If someone would manufacture them in a distinctive color, for example, it

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could be an attractive purchase. Avoiding over-tightening works as well, whether with the pencil or the proper tool. I've never broken a pencil in LN_2, but surely it's not the intended application. Michael Elbaum michael.elbaum@weizmann.ac.il

I found that a very nice replacement for the tool to handle the pin grid boxes are Pilot pens. They actually work better than the TFS tool. Ruben Diaz pindusito@gmail.com

I cool down the metal part very well with liquid nitrogen before using it. When the metal part becomes super cold, the metal holds the tip of the grid box cover well. When turning, I also push down the tool. If I do these there is no turning of the metal part without unscrewing the cover. Reika Watanabe reika.watanabe.c@gmail.com

Tips on Polishing Paraffin Wax Samples

Microscopy Listserver

I have the opportunity to do some very cool EBSD work on a fossilized eggshell (probably almost entirely calcite), which has come to me embedded in paraffin wax. I find that my normal polishing process is not going very well. I'm curious if anyone on this list has had experience with polishing a sample in paraffin, or if there is a safe way to remove paraffin from the equation entirely and proceed with a more traditional epoxy. The eggshell fragment looks cohesive, so I'm optimistic that it would survive a gentle removal of paraffin. Thank you all for your time and expertise! ***Omero Felipe (Phil) Orlandini omero.orlandini@jsg.utexas.edu***

I would remove the paraffin with a solvent, classically xylene was used in pathology but nowadays there are alternative solutions. Stephane Nizet nizets2@yahoo.com

The eggshell should survive removing the paraffin with no problem. I've done this with small crustaceans: fix, process to paraffin and embed, carve away the unwanted wax and crusty bits to expose the internal structures, de-embed to EtOH, CPD, and image in the SEM. Samples were fine. Might be an idea to practice on a chicken eggshell first. The protein matrix would be the most likely part to be affected by the treatments, and this should be less of an issue with fossilized eggshell. Any protein left should be robust. Polishing: I assume you're using a flat-lap. If so, the advice I was given was to make a figure 8, oriented vertically, so: lap wheel O, polish direction 8 (not on its side). And, if you're doing EBSD, you'll want to ion-mill your sample. Mechanical polishing won't give the best results for best EBSD. Phil Oshel oshel1pe@cmich.edu

Infiltration Issues with Chlamydia-Infected HeLa cells

Microscopy Listserver

I was wondering if anyone out there has experience working with Chlamydia infected cells and could offer some advice? I am having infiltration issues that seem to be localized only to the inclusion. It seems that there is material present in the inclusion that is difficult to embed. Does anyone have an idea of what this material might be, or any alterations to the processing protocol that might improve the infiltration? Papers I have found that use TEM to visualize Chlamydia infected cells use standard processing protocols, and we are using a fairly standard protocol here as well: Fix with 2% GA, 2% PFA in 0.1M cacodylate, 1% OsO_4 post-fix, ethanol dehydration, PO, 1:1 PO:resin overnight, and embedding the next day. Our resin of choice is EMBed 812, medium hardness. Any advice would be appreciated, thank you! ***Nicholas Conoan nicholas.conoan@unmc.edu***

Back in the dark ages (early 1980s), when I did my work for Brunham and Peeling, we worked mainly with cell monolayers. The problem is the structure of the Chlamydial bodies. In particular, the elementary bodies. They have a very thick cell wall, and the internal structures are very condensed. This makes them hard to infiltrate. The reticulate bodies, on the other hand, are a dream. My solution was to use acetone dehydration. Propylene oxide is not necessary if you are dehydrating with acetone, and in my experience, membranes were not leached out with the propylene oxide step. This also works with cell pellets. The only thing I cannot attest to is the success you would have with a tissue granuloma taken from an infected case, as I never worked with one of those. By the way, with acetone dehydration, rather than ethanol/propylene oxide, you do not need the uranyl acetate *en bloc* fixation to retain membranes. Leaching of the membranes seems to be associated with the propylene oxide, at least in my experience. Plus, you don't need the propylene oxide, thereby getting rid of a very noxious and flammable agent. Paul R. Hazelton paul.hazelton@umr.umanitoba.ca

Are you using vacuum infiltration? Jerry Jasso jfjasso493@gmail.com

Another option would be to use microwave-assisted processing and embedding (https://pubmed.ncbi.nlm.nih.gov/24357357/). Igor Kraev igor.kraev@open.ac.uk

Seconding and acknowledging Prof. Hazelton's post/opinion on the parameters to consider with these delicate 'critters'. A research paper that might help, and contains some slightly more detailed information (including acetone dehydration and intermediate fluid-infiltration) is Bradley et al., https://www.int-res.com/articles/dao/4/d004p009.pdf. From my knowledge and experience, I would add to Prof. Hazelton's proposals the following considerations/thoughts:

(1) prolong the processing time in most steps (especially fix, washing, infiltration);
(2) after osmication for 3-5 hrs (4°C; 2 hrs at RT in fume hood!), wash in appropriate buffer 2-3x for 5 min) followed by at least 1x 10 min 50% EtOH or Acetone;
(3) incubate in 1% paraphenylene diamine (PPD) in 70% EtOH or 70% Acetone. PPD in combination with OsO_4 'mordants' the specimen and retains lipids and 'matrix' substrate that is usually eluted by the standard dehydration protocol;
(4) wash at least 2-3 x (15 min each) in 70% EtOH or 70% acetone (until most of the brownish 'PPD-bleeding' in the washing fluid is removed);
(5) proceed with a further step of 70% EtOH pure or 70% acetone, and then continue with dehydration as usual (80, 90, 96, 96% each 5-15 min @RT, 100%, 100% (EtOH or acetone) at least 10-15 min each @RT;
(6) Intermediate fluid: resin mixture (hardener/catalyst/accelerator added) 1:1 only, at least for 2-3 hrs (@RT, specimen rotator);
(7) pure resin: 2-3 times;
(8) Polymerization: classically, I used a 3-step polymerization: (water vapor-free polymerization ovens): 24 hrs @ 37°C, 24 hrs @ 45°C, 24 hrs @ 65°-70°C. Wolfgang Muss wij.muss@aon.at

Phenol Red in the Imaging Medium

Confocal Listserver

I have heard that the reason Phenol red is avoided in cell culture media used for imaging is that it quenches fluorescence in some (at least the green) spectrum. I cannot find any reference about this. Does anyone know of a paper showing this? Thanks. ***Sylvie Le Guyader sylvie.le.guyader@ki.se***

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Try this paper: DL Becker et al., https://doi.org/10.1113/jphysiol.2007.138776. Figure 3 shows the decrement in DiI signal with and without Phenol red. Kevin F. Webb kevin.webb@nottingham.ac.uk

Thanks, Kevin. Yes, good stuff in Figure 3, but this is 840nm excitation. I wonder if the same occurs with single photon excitation. Anyway, this is a good one! Thanks! Sylvie Le Guyader sylvie.le.guyader@ki.se

If my memory serves me well, there was a chapter in Jim Pawley's book: Handbook of Biological Confocal Microscopy, Third edition: Confocal Microscopy of Living Cells, Michael E. Dailey, Erik Manders, David R. Soll, and Mark Terasaki, pages 381-403. Franco Del Principe franco.delprincipe@lis.ch

Phenol red seems to have a strong absorbance at 550nm only at alkaline pH (https://www.researchgate.net/publication/221925346_Plastic_Optical_Fiber_pH_Sensor_Using_a_Sol-Gel_Sensing_Matrix/figures?lo=1). But, if left at room temperature, bicarbonate-based media will turn alkaline, so it is possible that some quenching by energy transfer occurs. Mike Model mmodel@kent.edu

Phenol red (and other cyclic compounds) has been shown to increase background fluorescence (its peak is 440 nm), which can create issues for users depending on the channels being used. One such reference is https://link.springer.com/protocol/10.1385/1-59259-826-9:395. Hence, if using colors in that spectrum, it is best to use Phenol red-free medium. Additionally, Phenol red's cyclic nature is similar to that of estrogen, so it can bind to and activate estrogen receptors of estrogen-sensitive cells (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC323325/). If doing work in those areas it is also a good idea to remove Phenol red. John Fisk techsupport@thermofisher.com

The reference given by John from Thermo Fisher shows a 20% increase in background fluorescence in the green and red channel when using Phenol red compared to medium without. There is also information about estrogen which I did not know. Absolutely nothing about quenching fluorescence. However, I saw that the measurements were done on plastic dishes. I did measure background fluorescence in the green and red channels with and without Phenol red some years ago and never found any difference. I wonder if one would get the same results on glass bottom dishes. Pawley's confocal handbook (p. 361) mentions that the problem with Phenol red is fluorescence quenching but there is no reference. Sylvie Le Guyader sylvie.le.guyader@ki.se

My understanding is that the autofluorescence could also be due to riboflavins. I have noticed higher levels of murky autofluorescence when higher levels of fetal calf serum are used, particularly in the 488 channel, for example, GFP-expressing cells. This is a rather old flow cytometry paper, but I find it quite useful: https://journals.sagepub.com/doi/pdf/10.1177/27.1.438504. Please have a look at Figure 4. Jacqui Ross jacqui.ross@auckland.ac.nz

The main problem with Phenol red is usually given as autofluorescence. In its presence, cells and tissues show a diffuse fluorescence. Given that it appears red, we might also assume that Phenol red absorbs red and blue light. Which raises the possibility that Phenol red could differentially reduce the excitation produced by light in this range at increasing depths. That Phenol red absorbs light might mean that what is described as autofluorescence may actually be fluorescence from Phenol red associated with the tissue. This would explain why it is a larger problem with some cells type - T cells are mentioned. Apologies for not being able to reference this. Jeremy Adler jeremy.adler@igp.uu.se

Springer protocol: Stadtfeld 2005 in Methods in Molecular Medicine, Vol. 105: Developmental Hematopoiesis: Methods and Protocols: 2.3. Phenol Red-Free Medium and Glass Bottom Vessels Improve Image Quality. The second parameter that contributes to image quality is background fluorescence. To test the influence of Phenol red in the culture medium on background fluorescence, we acquired a series of images in media with and without this pH indicator. As shown in Figure 3, Phenol red dramatically increases the background levels, especially when visualizing GFP and RFP with the Endow GFP and TRITC filters, respectively. As a result, the relative signal intensity is decreased, hampering the detection of weak signals (when visualizing YFP with the Yellow GFP and JP2 filters, this effect is only minor). Therefore, we recommend using Phenol red-free medium (we routinely fill a chamber in an eight-chamber slide or a row in a 96-well plate with Phenol red-containing medium to visually monitor the pH of the cultures). "Phenol red dramatically increases the background levels"… Figure 3 shows under 20% increase in intensity. See Lambert et al., 2020: (https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.3000936). No need for Phenol red in culture media on a microscope, and bicarbonate ions are not just a buffer: they are a substrate for CFTR and several other ion transporters. Evrogen has been selling DMEMgfp for nearly a decade (URL is for DMEMgfp-2): https://evrogen.com/products/medium_DMEM_gfp/medium_DMEM_gfp.shtml and ThermoFisher and abcam (acquired Marker Gene Tech): https://www.abcam.com/opti-kleartrade-live-cell-imaging-buffer-1x-ab275938.html sell similar media. George McNamara geomcnamara@earthlink.net

Stable Stochastic Optical Reconstruction Microscopy (STORM) Buffer Reagents

Confocal Listserver

We have a Nikon STORM system at our facility. It is used extensively for short periods when a user needs it and then several months (or years) may pass before the next user pops up. When the time comes to demo STORM at a course, we often realize that our 'new' reagents have gone bad. Typically, mercaptoethylamine (MEA) buffer has absorbed water, despite the fact that we aliquot it in screw cap tubes in nitrogen. I assume that others are in the same situation. Does anyone have a solution? How long does MEA stay good and if it is more than 1 year, how should it be stored? I can see that there are new fluorophores that blink without oxygen scavenger buffers for live cell STORM, but as far as I know these fluorophores are not yet commercialized. ***Sylvie Le Guyader sylvie.le.guyader@ki.se***

We have had the same issues you describe due to the hygroscopic nature of the MEA. We typically make up our MEA at 100 mM in PBS (correcting the pH), and store aliquotted and frozen at -80°C. For fluorophores that require scavengers, we then make them up in a final concentration of 50mM. Imaging then takes place with sealed samples, excluding the atmosphere to increase their duration. We can get 6 months from the frozen MEA and 3-4 weeks for the samples. Colin Rickman c.rickman@hw.ac.uk

Has anyone tried the buffer detailed in this BioRxiv paper: https://www.biorxiv.org/content/10.1101/465492v1, which potentially gets around some of these issues (although probably not TIRF compatible). We are planning on giving it a go in the next few weeks. Simon Walker simon.walker@babraham.ac.uk

I have used even very old MEA stored at non-optimal conditions for successful dSTORM measurements in the past. Some amount of absorbed water is usually tolerable in my opinion - although it might make it more difficult if you need to know the exact amount of MEA in your buffer. However, many dyes are somewhat robust in their behavior when it comes to the concentration of MEA. Additionally, while not ideal, a dSTORM measurement without an additional oxygen scavenger system present is also possible with some dyes (like AF647). While this might not always produce images for your next publication, they should be fine for demoing dSTORM, or checking sample preparation protocols. You might also have a look at this paper for a different approach to the issue of oxygen scavenger systems: https://pubs.acs.org/doi/abs/10.1021/ac400035k. Patrick Then patrick.then@uni-jena.de

Although not suitable for live-cell STORM, your messages inspired me to look back at some papers reporting the use of commercial Vectashield as a STORM imaging buffer. I've previously used it with good results as a more stable training/test sample. However, I see a more recent paper reported that Vectashield quenches AF647 (https://www.nature.com/articles/s41598-020-63418-5). Simon, perhaps you can use sodium sulfite+thiol without glycerol for TIRF compatibility? Looks like that paper reported good results as well. Ben Hibbs ben@kleinaustralia.com.au

I agree with what most people have already suggested: 1. MEA can be aliquoted (solid or as 1M solution) and then frozen; 2. BME is another more toxic/stinky but more stable option; 3. MEA in sulfite is stable for longer than without sulfite; 4. Vectashield works well (I'm biased here), though the quenching is real, so it is a better option for tubulin samples than low abundance proteins. Diluting in 1/4 in glycerol rather than using it pure does help and I get good reconstructions (granted, on tubulin). Ko Olivier niko.olivier@gmail.com

Stimulated Emission Depletion (STED) Microscopy Blowing Holes in Mounting Medium

Confocal Listserver

I've got a user who is using STED to look at neurons cultured on poly-lysine-coated coverslips. They are fixed and mounted in ProLong Diamond. Some slides show holes or rings that develop during scanning, even with fairly low 775nm depletion laser power. I've imaged many ProLong-mounted slides with STED over the years with much higher power and never saw this before. Has anyone seen this, or have an idea what might be happening during mounting/curing to cause this? The ProLong appears to be cured (at least to some degree) since the coverslips are stable. Thanks. ***Chris O'Connell coconnell@uconn.edu***

Hard to say without more detail about the samples, but I have had rare samples where something similar has occurred. In particular, this was the case for malarial parasites and specific retinal samples. If your sample has any sort of chromophore or other highly absorptive component that is susceptible to absorbing the wavelength of the STED beam, then this can cause severe, localized damage to the samples. If you have any sort of plasmonic structures, this can also be an issue. If your STED has access to dynamic illumination schemes (DyMIN), then you can completely avoid this by automatically shutting off the STED beam when such extreme events are detected. Nicolai Urban nicolai.urban@mpfi.org

To add to what Nicolai mentioned, and assuming you haven't explored this already, you might check to see if the user is flaming the coverslips versus acid washing during prep. Flaming may leave some carbon deposits that can absorb and create holes like you describe. Acid washing is generally preferable here. Jessica Shivas jessica.m.shivas@gmail.com

I agree with Nicolai that this happens (only) if the STED depletion laser is absorbed by the sample. This can happen if the mounting medium has a slight hue in it, like Vectashield. We have also used ProLong and never observed this problem. Is it possible that your sample may have some staining residue present? If you use antibody staining, I wouldn't think so, but with histological dyes this could possibly happen. If the samples are checked under bright field conditions, it might reveal if dyes or carbon particles are present. Steffen Dietzel lists@dietzellab.de

Quality Control and Fitting of the Point Spread Function

Confocal Listserver

We image sub-resolution beads on our confocal microscopes and perform a 3D gaussian fit to determine the center and full-width half maximum (FWHM) in x, y and z directions. We noticed that on our Nikon A1R HD microscope, the point spread function (PSF) is elliptical at 45 degrees when using a 60X oil, NA 1.4 lens. I was told this is because no quarter wave plate is inserted so the best brightness and contrast can be obtained. This raises three questions that I would like to ask to the community: 1. Do others with a Nikon A1R HD also notice this effect with high NA objectives? 2. Can someone explain why the brightness and contrast is better without the quarter waveplate? I do not have a physics background, and this is hard to follow for me, but I would like to understand the reasoning. 3. Does someone know a FIJI plugin or other free software package that can do an elliptical (3D) fit to analyze the PSF for quality control so we can quantify the difference between the longest and shortest axes instead of comparing x and y? ***Herlinde De Keersmaecker herlinde.dekeersmaecker@ugent.be***

We have Nikon confocal microscopes and also acquire PSFs. Quite a long time ago we realized that the DIC slider (I assume that this is what you mean?) under the objective was making the PSF longer and a bit larger, but it was not at 45 degrees. Our policy since we realized this was to have no DIC slider by default. We keep them in an objective box near each microscope and instruct the users who require DIC how to insert all the DIC elements. Sylvie Le Guyader sylvie.le.guyader@ki.se

I would concur with Sylvie on the DIC prism. We have a couple of spinning disk and widefield systems that show a significant 45 degree elliptical PSF in the lateral plane when a DIC prism is inserted. We also have a Nikon SoRa system which shows a very small ellipticity, also at 45 degrees, using the 100x objective. The engineer suggested I tweak the correction collar to minimize this, which is something to try if you have one on your objective. As for software which can fit the elliptical shape. If I understand correctly and you mean elliptical in the lateral plane, then PSFj can fit this shape. All the other PSF programs I am aware of only fit the x and y directions and won't spot the elliptical problem. If you need full 3D fitting, then PSFj has an option to do this, although I have never used it myself. Claire Mitchell camdu@warwick.ac.uk

From a paper (https://doi.org/10.1117/1NPh.4.2.025002) a while back, Dr. Micu and I discovered that most confocal systems are linearly polarized. I suspect this allows them to work with various DIC optics, and the Nikon microscopes in particular offer

a laser scanning DIC modality that relies on linear polarization of the laser. Without taking a deep dive into the physics, I can tell you that linear polarization causes unusual interference effects that can manifest in an oblong PSF when the light interacts with an edge or curved surface. The way to avoid this is to circularly polarize the light such that there is no directional preference. If you dig a bit deeper into our paper, though, you will see that it may cost some fluorescence intensity depending on the fluorophore. Craig Brideau craig.brideau@gmail.com

I think that the DIC (Nomarski or Wollaston) prism splits PSFs by shear amount, which is equal to shear angle multiplied by tube length and divided by the objective lens magnification. I measured the shear angles for high contrast, general, and high-resolution Nomarski prisms for Olympus microscopes. For example, the shear is 70nm if one uses the high-resolution slider Olympus U-DICTHR and 100x objective lens. More measurement results and computed shears can be found in my chapter "Differential interference contrast microscopy". In: Biomedical Optical Phase Microscopy and Nanoscopy (2012), NT Shaked, Z Zalevsky and LL Satterwhite, eds, Elsevier. Of course, if the excitation light is linearly polarized and its polarization plane is parallel to the eigenpolarization of the DIC prism, then the beam will not split and there will be one PSF. However, if the polarization is not linear, or it is linear but oriented differently, then the DIC prism will separate the beam and split the PSF accordingly. Usually, the fluorescent light is not polarized, and we cannot avoid splitting of the emission beam by the DIC prism. Therefore, the prism should be taken out. To answer the question about a quarter waveplate, it is necessary to know its type (model), location and orientation. Michael Shribak mshribak@mbl.edu

Thank you for all the interesting and useful feedback and links. I went back to the microscope, and it was indeed the DIC prism (I should have thought about this). From one of the references, I understand now that the reason Nikon did not put a quarter waveplate in place is probably because we have a multiphoton laser. The program PSFj seems to do the trick, thanks for the suggestion! Herlinde De Keersmaecker herlinde.dekeersmaecker@ugent.be

Leica has a nice motorized DIC with a wheel underneath the objective turret so that the right prism is inserted when DIC is used, and the prism automatically removed when normal transmitted light imaging is used. Very useful for a core facility. I found that without the DIC prism, the polarized beam causes PSFs which are elongated in one lateral dimension. This was x for our Leica microscopes and y for the Zeiss LSM 780. Measuring the resolution from line profiles in x and y will give quite different results! Rotating the scan field will change the orientation of the long axis of the elliptical PSF. Andreas Bruckbauer a.bruckbauer@imperial.ac.uk

We'd like to add that asymmetrically shaped PSFs are not uncommon, even without accidentally having a DIC slider in the optical path. The possibility to produce a theoretical PSF with exactly the same image parameters as a sub-resolution bead facilitates quality control measurements of an experimental PSF. Our Huygens Professional software (available for a fee - sorry) can produce a high-quality theoretical PSF based on the same parameters as the bead image. FWHM measurements are performed on-the-fly and can also be measured in all XYZ directions. Subsequently, experimental PSFs can be derived from bead images using our Huygens PSF Distiller. The Distiller also reports the FWHM measurements of the experimental PSF. Lastly, images recorded with a system can be corrected by deconvolving them with this distilled PSF. Vincent Schoonderwoert vincent@svi.nl

In 2010 I found that one of our confocals had a PSF off by a few degrees. However, in more than 10 years we have not detected any problems, including with 3D reconstructions of biological samples, and I say "we" liberally because no other user, out of hundreds, has noticed the problem. Certainly, the users who insist on using the 10X NA 0.3 lens would never notice this. Michael Cammer michael.cammer@nyulangone.org

One Versus Two Disks for Spinning Disk Confocal Microscopes

Confocal Listserver

We are considering a new spinning disk confocal live imaging HCS system. Some come with Yokogawa (2 disks) and some with single disks. I understand the advantage the dual disk provides in terms of concentrating the excitation light through the bottom disk onto the sample, but is a laser-based single-disk spinning disk confocal still a good solution? Does anyone have a single-disk confocal they can comment on? Thanks. ***Irit shoval irit.shoval@biu.ac.il***

We have the Crest V3 from Crest Optics (single disk) and we also have a Yokogawa X1 (double disk) which has pinholes closer to each other than the newer W1 version. We are happy with both, and the advantages/disadvantages are not caused by the number of disks but more the microscope and the camera they are attached to. 1 versus 2 disks: 1. More powerful lasers are needed with the single disk design; 2. The system you choose should have an illumination homogenizer. Otherwise, the corner of each image will be darker, which will be visible when tiling. Sylvie Le Guyader sylvie.le.guyader@ki.se

I've worked with both the X1 and W1 instruments, and while I really liked the X1 design (no dust issues), the W1 delivers much better images. The increased pinhole spacing makes a difference. Crest was going to offer a single-disk setup with the W1-like pinhole arrangement, but I haven't heard about it recently. I haven't tested the Crest V3 extensively, but in some Crest designs there might be an issue with very dim samples. The autofluorescence from some elements common to the powerful excitation beam and the emission light might contribute to background (=noise). The best way is to demo the instrument, it's quite likely that it will perform just fine. Zdenek Svindrych zdedenn@gmail.com

We just posted to bioRxiv a manuscript (https://www.biorxiv.org/content/10.1101/2021.09.04.458950v1) on a "do-it-yourself spinning disk" that may be of interest to some readers of this list. (This is not a commercial post.) It is also somewhat relevant to the original inquiry about single versus dual layer disks. The short of it is that for those with a background in home-built instruments, one can design, purchase, and assemble a single-layer spinning disk module inexpensively ($1,000-$7,000 in our case) that can be integrated with an existing microscope setup that already includes lasers, chassis, objective lenses, filters, camera, etc. The disk pattern can be easily customized for a variety of objective lenses (air, oil, water, different magnification and/or NA) and applications (cells, tissue, expansion, sm-mRNA FISH, STORM, DNA PAINT). As stated by others, the

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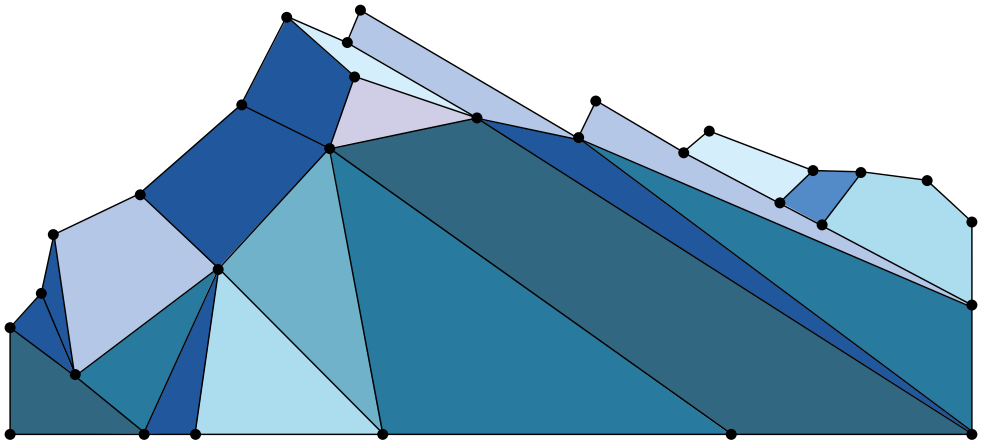

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single-layer disk indeed requires more laser power than dual-layer disks that include a microlens array, but lasers are often relatively powerful these days. Josh Vaughan jcv2@uw.edu

This DIY spinning disk is quite impressive. I was wondering, the commercial disks are made of identical sectors (I mean "angular" sectors, to distinguish it from the radial sectors in the preprint) - this helps with short exposures (there are typically 8 identical sectors). Is the multiple spiral approach somehow equivalent to this? My other comment goes to fluorescein. I've spent a lot of time with fluorescein, and… let me put it this way… we're not friends. There is a dramatic decrease of absorbance at 488 nm between 1 mM and 10 mM concentrations, as well as a drop in quantum yield to almost zero. It's quite possible that at 1M the fluorescence only originates from the coverslip surface (nobody really knows), but if you extrapolate the dilute fluorescein extinction coefficient, that's not right. And its sodium salt of fluorescein for that matter, as fluorescein is insoluble in water. Also, why didn't the disk vendor drill the hole to the typical engineering tolerances (10 µm should be possible)? Zdenek Svindrych zdedenn@gmail.com

Yes, the product number we referenced is indeed for the disodium salt of fluorescein. Do you have a literature reference on the absorbance behavior of fluorescein at high concentration? We could revise or remove our ~62 nm 1/e estimate, but the exact value is not important for our purposes. That is, the ~800 nm FWHM of the fluorescein signal we measured with the water lens matches the 100 nm-bead-measured axial PSF so the emission layer of 1M fluorescein seems thin enough for measuring background rejection out to +/- 10 µm. Is it 8 sectors per revolution in commercial disks? As to your question, we just didn't study partial revolutions since for most of our experiments we use >0.1 second exposures in multiples of a revolution. Fortunately, the photomasks are relatively affordable and can be easily customized for speed or other priorities. There may be more precise ways to mount the disk, but our solution seems adequate. We aren't sure about the tolerance for hole placement by the photomask manufacturer (it might have been as low as 10 µm for all we know) but we still think it is good to use a design that tolerates being off-center. Josh Vaughan jcv2@uw.edu

Regarding fluorescein absorbance, all the relevant research is very old, and I don't have good references. What's worse, I was *totally wrong* about the absorbance decreasing appreciably with increasing concentration at any wavelength. I was misled by our microvolume-spectrophotometer that reads zero when the absorbance is above ~50. I was getting double peaks like in Figure 5 of [https://onlinelibrary.wiley.com/doi/10.1111/j.1365-2818.2008.02026.x], just much deeper. Other references related to this figure might be of interest to you. If anything, the absorption increases faster than linearly with concentration, or at least shifts to longer wavelengths. You can see it readily when comparing 100 mM Na-Fl in 1 mm cuvette versus 10 mM Na-Fl in a 10mm cuvette, see, for example, this image (https://drive.google.com/file/d/1dwi18lhBxjC2fwq8hysDfwHgX8-Zi1S_/view?usp=sharing); the darker one is the 1 mm cuvette with 10x the concentration. I have no means to measure the absorbance other than the long-wavelength shoulder, which shifts to longer wavelengths with higher concentrations. I know, there are machines that can go to OD 6, I just don't have one. The quantum yield drop with increasing concentration is a well-known feature used in many "dequenching" experiments, see, for example, Figure 3A in (https://www.nature.com/articles/srep29460). Sorry for the confusion brought about by my previous post, I know it can't be undone and will remain in the internet's history for eternity. Zdenek Svindrych zdedenn@gmail.com

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Crossword Puzzle Answers

See puzzle on page 58.

1 D	2 I	3 S	4 T	5 O	6 R	7 T	8 S	■	■	9 G	10 L	11 A	12 R	13 E
14 E	S	T	I	M	A	T	E	15 D	■	16 F	I	B	E	R
17 P	R	I	C	E	■	18 Y	T	D	■	■	19 A	E	O	N
20 T	A	G	S	■	21 P	L	S	R	■	22 O	I	R	P	E
23 H	E	■	■	24 M	E	■	U	■	■	25 U	S	R	E	■
■	26 L	27 A	28 S	E	R	■	29 P	R	30 O	T	E	A	N	■
31 B	I	A	S	■	■	■	■	■	32 W	T	■	T	■	33 U
R	■	34 C	O	35 N	36 T	37 A	38 M	39 I	N	A	40 T	I	41 O	N
42 E	43 S	■	■	44 H	A	V	O	C	■	■	45 C	O	M	A
46 A	A	47 R	48 P	■	■	■	49 N	O	50 S	I	G	N	A	L
51 K	N	O	C	52 K	53 O	54 N	■	55 N	E	■	■	■	56 L	I
■	D	■	57 P	E	K	O	58 E	■	59 T	60 I	61 M	62 E	■	G
63 P	B	64 S	■	65 F	A	I	N	66 T	■	67 L	A	B	68 A	N
69 G	O	S	70 S	I	P	S	■	71 L	72 E	A	K	A	G	E
73 E	X	P	E	R	I	E	N	C	E	■	74 E	Y	E	D