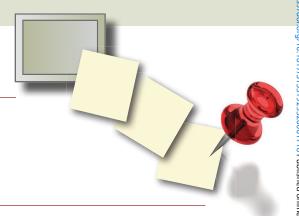# NetNotes

Edited by Bob Price

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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.

Centralized Versus Distributed Microscopy Cores
Confocal Listserver

Pretend for a moment that we are back in 2019, blissfully unaware of the COVID-19 related challenges that we face now. Later this week we will be discussing with our core administration the pros and cons of cores run by a single person. We have mostly a distributed collection of cores here by virtue of our sprawling land grant campus (a colleague's optical microscopy core is over 1 mile away and a round-trip is typically 40–45 min of travel time). To help us prepare, would you be willing to give us some feedback:
* What would you say are the strengths and weaknesses of centralized vs distributed microscopy cores? Other types of core services at our institution have centralized, but not microscopy.
* Since our discussion is mainly about personnel issues related to distributed cores run by one person, what pitfalls/problems would you highlight?
* Several of us at our institution have "been around for a long time". With money always being tight, any ideas for how to plan/prepare for our eventual replacement in a manner that best serves the users? Thanks! ***Doug Cromey dcromey@arizona.edu***

Generally, a centralized facility is more efficient from a staffing, organizational, and supply perspective. Though, in reality, the microscope often has to be located near the sample source. If the users are working with live animals, an *in-vivo* system located inside the animal care facility, or adjacent to a surgical suite, can be important. Even in the case of a tissue bank, having the imaging system located proximal to the tissue storage and preparation areas can be highly beneficial. Much of this depends on the layout of your facility, but most campuses tend to be fairly sprawling. This makes such centralization difficult, so it is often necessary to provide many individual machines. Craig Brideau craig.brideau@gmail.com

We are one facility but have two sites located about 4 miles apart. So, this means we usually only go to one of the sites and maybe visit the other in the afternoon. I think having people move between sites is good for holiday and sickness coverage. But an advantage of having dedicated people for one site is that they know the research groups and projects very well so the support can be more dedicated to the individual research needs. This poses the question: What is a facility? A selection of instruments? A group of dedicated people who run them? A set of rules for use, charging, etc.? An entity some scientists or the institute's administration creates out of thin air (or on available funding)? Andreas Bruckbauer a.bruckbauer@imperial.ac.uk

l am working remotely (with the facility still open to users) during the pandemic. This has shown that if users are well-trained and the equipment is well-maintained, most help can be delivered remotely. This is because the vast majority of the help we provide concerns software problems, sample prep advice, experimental design, and microscope setting advice. All this can be delivered remotely. In case of a (rare) hardware problem, we have to be there physically. Sylvie LeGuyader sylvie.le.guyader@ki.se

We had a long discussion about this in Oct. 2019, essentially training button-pushers versus creative experts. There was no consensus. Different labs do things very differently. A majority of our training regimen is of the button pushing type. People want answers fast, don't have the tolerance or interest for longer training, or want to keep costs down. (Today someone wrote to me contrasting data from a Leica confocal with data from a "compound microscope," in fact an Apotome. At least I wasn't the person who did the training.) Some users are already microscopists and others do become experts. Some crave knowing more and we will teach all we know until they know more than we do. But to repeat, most are satisfied with and/or are only tolerant of the most cursory training to get them the minimal pictures they need to satisfy their project. But this wasn't the question. I replied to the original question with a much longer rambling email. I will try to summarize here. Having a core with one staff member requires substantial management of expectations to protect the staff. For instance, one person cannot always be available, and clients must understand this. Unless the staff likes to work all the time, uninterrupted evenings, weekends, and vacations need to be managed. If someone is on call when traveling, the institution needs to pay for the technology for this. When staff is training one user, another cannot demand immediate help. When I was sole proprietor of a core, it was very difficult to leave the lab for even an hour. And now, with equipment scattered all over campus, we have issues of quality control and when people need help, even expert users, sometimes it is difficult to provide it immediately. A centralized core with maybe a satellite instrument here or there is the way to go. The original question discussed a pre-COVID or non-COVID world. Our facility is doing in-person trainings. We have eased into it since June, but we are now doing it with masks, glasses or eye shields, and clean gloved or sanitized hands. One modification is teaching the software part of running the microscope by Zoom. This may remain SOP because it is better than over-the-shoulder. Therefore, my call for centralized cores stands even in our current COVID world. Michael Cammer Michael.Cammer@med.nyu.edu

Power at the Sample
Confocal Listserver

*I've been contacted by someone in our faculty who needs to estimate/ calculate the power density of the fluorescence light source at the sample. He knows the lamp power and the objective lens (water lens) and NA. We also know the field of view. I've looked online but haven't found a method for working this out as yet. Can anyone help? Kind regards, **Jacqueline Ross jacqui.ross@auckland.ac.nz***

The absorption of the microscope optics, and losses due to the exact way the light source is coupled into the system, can lead to widely varying actual power levels at the sample. Typically, I would measure this with an appropriate power meter at the sample plane to get a precise number. Trying to estimate the losses is probably a losing battle and could put you off by an order of magnitude in some cases. Craig Brideau craig.brideau@gmail.com

I was thinking the same actually. Excuse my ignorance but what kind of power meter would we need? Something similar to what is used for measuring laser power at the objective lens? Jacqueline Ross jacqui.ross@auckland.ac.nz

Yes, those will work, but you need to consider two things: Lamp sources are broadband, and most of the "at the objective lens" sensors are silicon-based, which have a specific wavelength response. You typically set the wavelength on the meter to the wavelength of your laser to get the correct measurement. Since the lamp will be broadband, the meter will only be usable if you are band-pass filtering the light. This is typical for excitation filters in most fluorescent regimes, but not effective for white light illumination. Since you mention fluorescence in your message, I assume your user is working with a particular excitation band. Simply set the wavelength of your power meter to the middle of the band and you should get a reasonably accurate reading. For instance, if the excitation filter is 490/20 (490 nm centered in 20 nm bandwidth) then set the wavelength of the meter to 490 nm. The second consideration is that the spot from the lamp will be larger than a laser spot since it illuminates the entire field of view. Your sensor will have to be large enough to collect the entire spot. It is also recommended that you do not place the surface of your power meter directly at the focus, so you will want to defocus the point of light on the meter rather than having minimum spot size. The reason for this is that a large-area silicon detector can give incorrect results if the majority of the sensor surface is not illuminated: It is better to spread the power out over a wider area of sensor so that it is evenly distributed rather than having a single high-intensity point in the middle of the active detection area. Note this also applies for measuring lasers; do not put a focused laser spot on your active sensor area. Craig Brideau craig.brideau@gmail.com

Consider a power meter like this: https://www.thorlabs.com/ newgrouppage9.cfm?objectgroup_id=3341. It will come in quite handy as there are a variety of sensors from which to choose, including a slide sensor (S170C) which would suit your needs well (other sensors are designed for measuring at different locations of your optical path). Cvic Innocent ci43@cornell.edu

If you're using an arc lamp for a widefield system, my personal preference is a fiber-coupled spectrometer with integrating sphere (or other sampling accessory), calibrated to a NIST-traceable light source. It is fascinating what you learn about the transmission properties of your filters and other components of the microscope when you start poking around with a spectrometer. Checking all the filters in the filter cube for cross-talk has helped us to identify the source of strange signals in several cases. Silas Leavesley leavesley@southalabama.edu

I appreciate the clarification. Yes, the filter set is a bandpass on the excitation side, emission is LP. I will give this a try. Jacqueline Ross jacqui.ross@auckland.ac.nz

The integrating sphere and spectrometer Silas mentions will give very detailed information of the instrument spectral response, but the equipment can be expensive. The key advantage is it can record the absolute spectral power density response for broadband sources. For lamps with narrow filters and laser sources it is probably overkill, and a power meter like Cvic mentioned will be sufficient. In full disclosure, Dr. Pina Colarusso and I assisted Thorlabs with the design of the model Cvic mentioned. We both received free prototypes from the company as thanks for our help with the details. When I use them, I try to fill the sensor area by about half to two-thirds with the circle of light from the objective. Fiddle with the focus while watching the reading and it will be fairly steady when you have good sensor fill. Be careful not to contact the surface of the sensor with the tip of the lens as the sensor uses a thin coverglass similar to a coverslip to properly material match the refractive indices. It is water- and oil-proof so you can use the medium your lens is designed for directly on the sensor for a more accurate reading. It cleans up with alcohol and lens tissues, and works fine with air lenses as well. I use mine with 1.1 and 1.25 NA water-dipping lenses and 1.4 NA oil. Don't forget to set the correct wavelength on the control box before recording the power. Craig Brideau craig.brideau@gmail.com

I totally agree with everything Craig said. Just wanted to add some further points. If your microscope is equipped with a field stop and aperture stop, you'll want to make sure they are opened to the correct position. I'd assume that for your application you'll want the aperture stop opened all the way (for maximum power and irradiance at the sample plane). The field stop position will be dependent on what your desired field of view (FOV) is. Just open it up until the aperture lies just outside the FOV when looking through the eyepiece at a screen (the sample will have to be in focus in order to do this). The field/aperture stop may need centering and can be done so using an Allen key. If your system doesn't have a field stop, then you'll need to get creative to limit your illumination so that it is within your FOV. I've been able to do this successfully by sticking a precision pinhole onto the entrance of an integrating sphere and positioning this such that the pinhole lies at the sample plane. Another thing to note is that the light source will have to be configured for Köhler illumination. This isn't a concern if you're dealing with a light source that connects to the microscope via a LLG / LLG epi-port. There's plenty of literature on configuring most light sources for Köhler illumination. To convert your power measurement into irradiance, simply divide by the area, which is equal to the following: Area [mm²] = pi * (FOV diameter [mm] / 2)². If you don't know the FOV of your system, or would like a sanity check, the FOV diameter can be calculated using the following equation: FOV [mm] = f.n. [mm] / M, where f.n. is the eyepiece/camera field number and M is the magnification of the objective. If you want some further reading, we've created a white paper on measuring irradiance: https://www.coolled.com/wp-content/uploads/2019/10/Measuring-illumination-intensity-with-accuracy-and-precision-final-1.pdf. Alex Gramann alex.gramann@coolled.com

Good points! In theory Köhler should also provide even illumination if configured correctly as Alex says. You can verify this by putting a pinhole into a piece of foil and covering your sensor with it.

Move the pinhole sensor across the spot and you can check for spatial intensity variability. Craig Brideau craig.brideau@gmail.com

The S170C Craig mentions is a useful detector. The downside is the S170C won't accept high angle rays from immersion objectives. You can get around this by estimating the transmission efficiency for an immersion objective relative to a lower NA dry objective. (This is working from memory, haven't done this in a while). Focus a low NA lens on a clean, cover slipped slide, setting Köhler illumination, then collect a camera image. Switch to an immersion objective, reset the condenser and collect another image. The ratio of intensities between the 2 images approximates transmission by the immersion lens. Add filters to the light source for spectral behavior. I had a 75 mm x 25 mm metal holder machined to hold the S170C. A frosted plastic disk, about US$0.50 from a local plastics suppler, of the same diameter as the S170C, could be dropped into the holder as a target for focus and alignment of lens or condenser. Then the disk was replaced by the S170C for measurement. One could also use a Chroma fluorescent slide for fluorescence. Glen MacDonald glenmac@uw.edu

Hi Glen, when I worked with Thor on the design for the S170C sensor, one of the key issues was ensuring that it *would* work with high angles. I was dissatisfied with existing sensors with limited angles and worked to solve this explicitly. There is a special index-matching gel under the coverslip to ease the light into the silicon detector. If you want to calculate the maximum NA, calculate the Fresnel equations for Air/Water-to-glass depending on what medium the lens is using. An oil lens of course doesn't need to worry since the medium index is the same as the coverslip. You'll find the error is quite small up to very large NA even for air lenses. The Brewster angle air-to-glass is around 57 degrees at which point you are looking at approximately a 5–10% measurement error, depending on the polarization of the light out of the objective. Practically most air lenses will be much less than this. With high NA water and oil lenses, you will of course have a large measurement error unless you put a drop of water or oil on the sensor (the S170C is designed for this) and measure through the medium. The sensor is designed for this and so you can get the proper measurement. I've found empirically for NA > ~1.0 you will notice a significant difference. Try it for yourself; get a high NA oil lens and measure the power coming out of the objective without oil on the sensor. Then add a drop of oil, dip the lens in, ease focus back to enlarge the spot size on the sensor while maintaining the oil link between the tip of the lens and the sensor. You will get a higher power reading as more light will properly enter the coverglass thanks to the presence of the oil. You can also do this with a drop of water for water lenses. Just be sure not to break contact of the drop of medium between the tip of the lens and the sensor, and don't accidentally ram the tip of the objective into the fragile sensor face. After using oil, clean the sensor with isopropyl alcohol or methanol and a lens tissue. After using water just wipe it off with a lens tissue. Craig Brideau craig.brideau@gmail.com

Craig, thanks for the background. Sorry, I realize that I misspoke (mistyped?). I was using the S120C. For some reason I typed S170C, despite having the Thor page open for the S120C. Unfortunately, the S170C came out after I already had purchased the S120C and couldn't justify another sensor, no matter how great that design is for working with immersion lenses. I started out with the S121C, which is the same as the 120 but has lower sensitivity so that it can handle higher power levels. It was good for measuring lower-power Ti:Sapph beams prior to the scan head, after power stepdowns. Of course, you want to use a multi-watt thermopile for measuring the laser itself, but usually by the time you have it at the scan head it is <500 mW, which was the limit of the 121. You can also use the 120 and 121 to measure the collimated beam out of the objective turret by removing the lenses, as this eliminates the NA issue. Be sure to wear eye protection when doing this though, as any reflections of a collimated beam risks sending substantial optical power flying around the room. The fluorescent ring around the sensor area helps you find the NIR laser beam while wearing appropriate NIR blocking goggles. This also assumes your beam diameter is less than that of the sensor (<10 mm), or at least the beam as limited by the back aperture of your objective. A good trick is to use a foil hole to emulate the back aperture of your objective and place it over the sensor to estimate the power actually entering the lens itself. You can also do this with the 170 (and handle wider beams), but the 121 has higher power handling capability. Glen MacDonald glenmac@uw.edu

Tetraspeck Beads
Confocal Listserver

Are there any fluorescent beads analogous to Tetraspeck beads available? I have also found GATTAbeads. Is there anything else on the market? Thanks. **Petro Khoroshyy khoroshyy@gmail.com**

From the long list of Spherotech products here: https://www.spherotech.com/2020-2021%20Price%20List.pdf exactly two products seem like a good alternative to TetraSpeck beads: FP-0257-2 Fluorescent Particles, Multiple Fluorophore, 0.2%w/v, and 0.1–0.3 μm URFP-02-2 Ultra Rainbow Fluorescent Particles, ~1E10/mL, 0.1–0.3μm. Has anyone tried to look at these under a microscope? Small TetraSpeck beads (0.2 μm) are quite dim and it would be great to have a brighter alternative. I do have a good source of very bright 0.3 μm beads (my favorite dry erase markers), but these are single color only. Zdenek Svindrych zdedenn@gmail.com

Can you share more info about dry erase markers? Thanks. Petro Khoroshyy khoroshyy@gmail.com

You can grab a marker, draw a thin line on a coverslip, and look at it under the microscope. If you want to get really scientific, here's what I've tried: To get rid of any glue, and possibly free dye, I resuspended some beads in IPA (isopropanol). You don't need to cut the marker open, just dip the tip in a 1.5 mL Eppendorf tube filled with IPA. Wash several times with IPA or ethanol (spin at 14000 x g for a few minutes, discard the supernatant and resuspend in new solvent by vortexing or sonicating). If you suspect a lot of aggregates you can spin for a shorter time and keep the supernatant. You can then use the alcohol prep, or spin and resuspend the pellet in water. To make the beads stick to glass add a little bit of divalent metal salt to the water prep (like 1 mM $MgCl_2$). The beads are chemically quite stable; I've tried mounting them in aqueous media as well as LOCA (the UV-curable glue for fixing smartphones) or Sylgard (two-part silicone, RI = 1.42, usable with autofocus systems). The size is probably slightly below 300 nm and they are very bright. A crop of a z-stack with a 60x lens can be found here (sparse sample): https://drive.google.com/file/d/1DmICU1tRuLXINoOmLSgEOFj-4E5UQY50 (quite cool how Google preview copes with 16-bit multi-plane tiffs). I almost forgot the top secret ingredient: Expo Neon dry erase markers (https://www.uline.com/Product/Detail/S-22783). Fun fact, the blue marker fluoresces in the far red! I only wish they made a "white" variant as well. Zdenek Svindrych zdedenn@gmail.com

If you just want to measure PSFs and don't necessarily need all colors in one bead you can take highlighter pens to create a sample. Many types of pens work for beads with 300 nm diameter or more. If you

want to be on the subresolution side (for example, to measure high NA objectives) the only ones we have found suitable are the Zebra Mildliner pastel pens (Zebra Pen product number 78105). We had a poster on this at FOM 2019 and a workshop at TIM 2020. You can find all currently available information on this project (including sample prep) here: http://webdav.tuebingen.mpg.de/LM/FOM2019. If you have further questions please let me know. christian.liebig@tuebingen.mpg.de

Contact Spherotech directly and ask for FPMAS in suspension with what you want in suspension for flow cytometry. George McNamara geomcnamara@earthlink.net

I use micromod sicastar-F silica particles that withstand organic solvents (for clearing). They make 10 nm to 1.5 μm; single colors only. https://www.micromod.de/en/produkte-22-fluorescent_sil.html G. Esteban Fernandez g.esteban.fernandez@gmail.com

I love Esteban's suggestion: products including particles down to 10 nm!!! Not as small, and a lot more fluorophores, are Spherotech's FPMAS "9plex" beads (9 fluorophores, some FRET between, 3 μm diameter. These can be used with low NA objective lens to emulate super-res stuff. They also have other products with fewer plex. https://www.spherotech.com/flu_par_sli.htm; FPMAS-30M9 Fluorescent PMMA Particle Slide, UV, Light Yellow, Yellow, Nile Red, Pink, Purple, CyBlue, Sky Blue, and Jade Green. George McNamara geomcnamara@earthlink.net

Yes, in Esteban's post the number of provided sizes is impressive. Unfortunately, they provide white particles only as a sample on glass. I would prefer some in solution. Zdenek Svindrych zdedenn@gmail.com

Protective Window for a Confocal Microscope
Confocal Listserver

Dear All, I am considering a purchase of a protective window for our Zeiss LSM800 and I would like to hear advice whether this is a good idea, and if yes, which one to buy. The reason is our LSM800 is on an inverted stand and we already have oil leak into the Optovar. This was despite my regular checks of objectives (both sides) for leaking oil and extensive education of users on how important it is not to use too much oil and to clean lenses, especially on inverted stands. The Zeiss Observer has a dummy slider with an opening for 32 mm filters. I hope a protective window there will stop the oil.

1) Do you think using a protective window for a confocal microscope is a good idea or will the imaging be affected? This would be in infinity space, so I guess it should not be detrimental; 2) What characteristics of a protective window should I take into consideration (substrate, thickness, coating, flatness, surface quality, parallelism)? Currently I am inclined to use protective glass by Edmund Optics such as a 1 mm thick MgF$_2$-coated Sapphire window which has high transmission, or alternatively a 2 mm thick MgF$_2$-coated B270 window which has slightly poorer transmission but better surface parameters (and is much cheaper). Best regards, Tomasz Wegierski confocal@llmcb.gov.pl

I would highly recommend the Aqua Stop system from Zeiss. It is overpriced for some pieces of plastic, but it forms a very tight seal and pays for itself many times over. It is far superior to hair elastics/ glove fingers/condoms which we used in the past. Doug Richardson ds.richardson@gmail.com

Maybe I misunderstand the issue, but if it is because of oil getting into objectives, I use ponytail elastic fluffy things on every oil objective.

I don't understand why Doug does not like them. Doug, maybe you did not use ones that can soak up oil well enough? Just stretch one a bit and place it on the objective. It will soak up any oil and when saturated, just throw it out. Every month or so I switch them out and I sleep soundly - I have zero oil spilling off of the objectives. The Amazon link shows what I mean as it took me some minutes to find that exact type. I luckily found a package with perfectly sized ones in my daughter's drawer. https://www.amazon.com/Elastic-Bands-Rubber-Ponytail-Holders/dp/B075FLPVYT Avi Jacob avijacob@gmail.com

Aqua Stop works well for big spills when installed correctly and has proven effective on some of our Zeiss scopes. But we did have a user who spilled over or around it on our Zeiss 710, so it is not foolproof. Hair elastics are great for the oil. Michael Cammer Michael.Cammer@med.nyu.edu

Women in the US will recognize "ponytail elastic fluffy things" as "scrunchies," which you can buy under this name from Amazon and other suppliers. We use them also. They are helpful in a multi-user environment where inexperienced users can get overly exuberant in oiling objectives. They should be replaced when oil drips on them. John Lemasters JJLemasters@musc.edu

The hair ties work great with objectives that have enough of a neck for the tie to sit on next to the barrel. Our 40X objective (most frequently used) has a very small neck so the hair ties don't work. I regularly (bi-weekly) take the objective off, store it in its case upside down, and put it in a 37°C oven. It is ridiculous, in a bad way, how much oil comes out. The 40X also doesn't have a "lip" that acts as a bit of a reservoir (unlike the 60X and 100X objectives). On one of my heavily used systems, I have to go so far as to dismantle the top (stage, objective turret, and fluorescent turret) to get to the piece of glass that prevents leakage into the body (amazingly awesome feature of the stand). This takes 15 minutes although I shouldn't have to do that. Gary Laevsky glaevsky.lists@gmail.com

In the "old days" in the core we used to cut filter paper and then bore a hole with a cork-bore slightly smaller than the lens and place slide 2–3 (or as many as will fit) layers of the filter paper over the objective. This will not stop a "flood" of oil (which sounds very possible for some), but it will hold much more than you would think. The most important feature of this little trick is that the oil will show very quickly on the paper and hopefully will trigger a response. Some of the darker filter papers will show the oil better, but this will vary by manufacturer. No commercial interest here, just trying to protect all the optics in the system. Michael Stanley mstanley@chroma.com

Just my 2 cents. Scrunchies work well but are not foolproof. Cotton, not polyester, materials are more absorbent. In addition to scrunchies I suggest wrapping the lens barrel near the nose of the objective with plumber's tape to prevent oil from seeping into the barrel. It can be done so that the lens retraction still functions. Then once the lens is on the nosepiece of the scope the plumber's tape can be wrapped there as well. I've also used thin rubber sheets or parafilm as well as filter paper (thanks Mike S.!), to draw oil away from the lens and nosepiece. The best method, however, is to teach users proper oil application and ensure they clean up after themselves. Vickie Frohlich victoria.frohlich@stjude.org

I have to second Doug's recommendation for the Aqua Stop from Zeiss. We use this on both of our inverted confocals and I have found it to be the most robust protection I've ever used. In our core it has held

up to exposure to oil for well over 2 weeks while I was away on vacation and before I had a chance to inspect the system after returning. The difference between the Aqua Stop and other solutions such as hair ties or scrunchies is that the latter only works for small amounts of oil over very short periods of time. If you have a user that over oils for one imaging session lasting an hour or so these scrunchies will work if you immediately clean up. They fail to stop oil penetration over long periods of time with multiple users. Oil that gets down into the optivar is from chronic over-oiling and a scrunchie is no match for that. Under normal conditions I regularly inspect the instruments and even after exhausting levels of training some users just don't get it, are careless, or just plain don't care. So, we do our best to regularly inspect and Aqua Stop has prevented more than a few situations where oil could have ruined our objectives. Good luck! Jason Kirk jason.kirk@bcm.edu

Does this Holy Grail of oil protection (Aqua Stop) fit non-Zeiss stands? Gary Laevsky glaevsky.lists@gmail.com

Thank you very much for so many responses, although the discussion went a bit into a direction that I did not anticipate. But yes, it is all about preventing oil from getting into the microscope. Our 40x/1.3 Fluar lens takes a lot of oil and I frequently have to clean oil from its bottom side. In contrast, I have not seen any oil on the back of our 63x/1.4 even though it is used as frequently as the 40x. I have to admit I have been skeptical about hair elastics, because when they become saturated with oil they will just let it drip. IMHO, it is replacing the "oil cleaning" approach with an "oil buffering" approach. The "cleaning" approach has worked for us for many years. It failed when an inexperienced user (who apparently did not take seriously what I was talking about during training) made two mistakes. The lens was not cleaned and allowed to stay in the optic path after the work instead of being changed to the 10x objective, and it was a holiday so the system was not used for the next few days, giving the oil plenty of time to leak into the optivar. So, it was a combination of several factors, but it can happen again.

I think, hearing so many positive opinions about hair elastics, I will give them a second chance in our facility. Aqua Stop in my opinion is overpriced, and we do not currently do any experiments under perfusion to justify such purchase (this may change in the future). However, I would not like to give up on the protective window strategy. I think placing a glass element in the infinity space is not unusual. It would function as the last stronghold against the oil. And it would be considerably cheaper than Aqua Stop. I would of course test PSF. If PSF is not affected, I guess the protective window is OK with imaging. So, if you still have some advice which one would be most suitable, I would appreciate it. Tomasz Wegierski confocal@llmcb.gov.pl

Back to your question. The filter holder is there to hold a filter. Any decent glass or fused silica AR window should do the trick. As far as I remember, the hole is quite big, around 30 mm diameter (bigger than the standard 25 mm filters), which may limit your options. Most filters and windows have specified surface flatness (reflected wave error), but it's actually transmitted wave error (TWE) that matters here. Usually, windows with single digit flatness (in lambda or fringe units) will have TWE << 1/5 lambda, and should be OK. Thicker substrates (2–3 mm) usually mean precision optics and is a safe bet. A hard coating is a must, if you want to wipe oil off of it regularly. All reputable brands use hard coatings these days, 1% loss (per pass) is OK. Sapphire is hard to work with and comes with a price premium. Fused silica, for example, https://www.edmundoptics.com/p/30mm-dia-vis-nir-coated-1lambda-fused-silica-window/10158/ (no commercial interest) looks like a good option, but BK-7 will work well, too. If you happen

to have a piece of AR-coated glass at hand, just put it there and look at some beads. If you're not able to tell the difference, you're good to go. You can even try a glass coverslip, the 4% loss (the excitation loss is not an issue, you can always crank up the laser) will be hardly noticeable. Coverslips are not polished, so the TWE is generally bad, but with some luck you can find a good one in the stack. Zdenek Svindrych zdedenn@gmail.com

Staying on the question, with regard to putting a piece of glass in that slider (which would make our lives easier), should it be placed on an angle, to minimize reflections? But, will that increase aberration? Good question, Tomasz. Gary Laevsky glaevsky.lists@gmail.com.

Colleagues, given that the protective window would be on a slider -- how difficult would it be to move it into and out of position? Perhaps for critical optical applications, it would not be too difficult to slide it out of the beam? Stray thought. Tobias Baskin baskin@bio.umass.edu

Most microscopes contain some sort of angled beam splitter or dichroic filter in the infinity space. And, indeed, most flat surfaces (windows, filters) are angled. Zdenek Svindrych zdedenn@gamil.com

Following the advice of Zdenek (thank you!) on the importance of TWE I decided to order this: https://www.edmundoptics.de/p/30mm-dia-4mm-thick-vis-0deg-coated-lambda10-fused-silica-window/7997. Its transmitted wavefront is lambda/10, and it shows very good parallelism, surface finish and transmission for VIS light, which is sufficient for us. I guess the opening for filters in the filter slider is angled, but I will check this. And the slider can be removed at any time from the optic path. It will be very easy to check imaging with and without the protective window. When I test the PSFs I will let you know about the results. PS: in my previous mail I meant 40x/1.3 EC Plan-Neofluar lens rather than "Fluar," which is a different lens (and less suitable for confocal). So, I meant one thing but somehow wrote the other. Sorry for that. Best, Tomasz Wegierski confocal@llmcb.gov.pl

As promised, I can now give some feedback on the protective window: I purchased the item #47-838 which is a 30-mm VIS-coated silica window with the following parameters: thickness 4 mm, parallelism <5 arcsec, surface quality 20–10, and transmitted wavefront lambda/10. 1) The opening in the filter holder is tilted, so the protective window after installation is also tilted in the optic path to minimize reflections. 2) The protective filter is 30 mm in diameter, which is a bit smaller than the filter holder (32 mm). To make it sit tight I will have to order an appropriate O-ring. Only then will the protective window provide full protection against dripping oil. But even without the O-ring, the protective window is larger than the aperture of the filter slider, so it sits nicely in place. 3) I checked the PSFs of 200 nm beads, and I cannot tell the difference in images with and without the protective window. I could not measure resolutions because I slightly overexposed the PSFs, but PSF shapes in xy, xz, and yz planes looked the same to me. 4) I noticed a slight increase in lateral shift between the green and far red channels: from ca. 30 nm to ca. 50 nm. This is a bit surprising to me, as there should be no bending of rays, and besides the Abbe number is not too bad (67.7). I did not the check green vs. red channel because I do not have a proper sample at the moment. In summary, I think the protective window is suitable for confocal imaging. For critical applications, it can be easily removed from the path. I feel much safer when this protective window is in the path, especially when I am not at the institute because of the COVID-19 situation. If I discover any more disadvantages of this protective window I will let you know. Tomasz Wegierski confocal@llmcb.gov.pl

Tomasz wrote: "4) I noticed a slight increase in lateral shift between the green and far red channels: from ca. 30 nm to ca. 50 nm. This is a bit surprising to me, as there should be no bending of rays, and besides the Abbe number is not so bad (67.7). I did not check green vs. red channel, because I do not have a proper sample at the moment." I would expect any tilted optic in the light path to cause some lateral chromatic aberration, even for low dispersion glass. The only way to completely eliminate this would be to have an identical optic in the same path tilted the opposite way. Still, 30–50 nm is a rather minimal shift and many microscopists could live with that for the sake of protecting the internals of the microscope. Craig Brideau craig.brideau@gmail.com

Beetle Wing Elytra
Microscopy Listserver

I have some beetle wing covers (elytra) to embed and section for TEM. Anyone have success with this? A typical embedment in EMBed812 (Epon) with a medium hardness is not holding the wing in the section. I'm thinking either a harder formulation, or a different resin. I don't believe fixing it like tissue would make a difference, however I'm not an expert in insect cuticle preparation and sectioning. Thanks! **John Shields jpshield@uga.edu**

Insect exoskeleton is mainly made of chitin. To soften the chitin, after fixation, place the specimens in a liquid such as Lysol or Simple Green Clean Finish that contains alkyl dimethylbenzylammonium chlorides for 20 min. to 1 hour. No kidding! I use it for drosophila eyes in paraffin sections. This works in the same way it kills bacteria, fungus, and viruses. Paula Keene Pierce paula@excaliburpathology.com

Adding 1% Z6040 to the ethanol during dehydration functions as a coupling agent to bind the resin to the cuticle, and I can confirm it works on insect wings. This is explained in more detail in my recent M&M2020 article: "Utilization of Z-6040 Organosilane as a Coupling Agent to Improve the Adhesion of Epoxy Resins to Waxy Biological Tissues" DOI: https://doi.org/10.1017/S143192762001781X Joe Mowery joseph.mowery@usda.gov

I would recommend adding Z6040 to the resin mixture. I've used Z6040 in epoxy, LRW and HM20 (100µl/10 ml resin). It will greatly improve the sectioning. Karen Kelley vau@ufl.edu

Hexamer Dissociates While Freezing Cryo-EM Grids
3D Listserver

I would like to ask the community for suggestions on how to overcome the issue of hexamer dissociation while freezing cryo-EM grids. With negative staining there seems to be a perfect hexamer at 0.1 mg/ml concentration, but the sample seems to fall apart at 1.5 mg/ml when cryofreezing. Please advise. **Umar Farook umarfarook12@gmail.com**

Your sample might be destroyed by the air/water interference. To remedy this try using detergent. Jay Rai jrai@fsu.edu

I agree with Jay. Using detergents or surfactants (octyl glucoside 0.1% final conc. or fluorinated octyl maltoside 0.0125% final conc.) might help you. The detergents repel the protein from the water/air interface. Be aware that this makes the ice thicker and you need a much higher concentration of protein as compared to blotting without detergents. If blotting *per se* (the force applied by the contact with filter paper) is the problem, you could try to fix the hexamer with mild

glutaraldehyde treatment prior to blotting. We occasionally use 0.5% (v/v) glutaraldehyde (EM grade) and incubate for 10 min at 4°C and then immediately blot and plunge-freeze. If everything fails, then you might want to try blot-free techniques to apply the protein to grids (for example, spotting or writing). There are commercial machines around like the Chameleon or VitroJet systems, or you can try to spray the protein (see S. Muench lab or J. Frank lab, and others). Magdalena Schacherl magdalena.schacherl@charite.de

Another option is to use GO grid then you can stay at the same concentration (+/-) as the NS and you also don't have the air/water interface. Elad Binshtein elad.binshtein@vanderbilt.edu

Or if you already know that your protein is happy on a surface you might want to give graphene or graphene oxide grids a try. Felipe Merino felipe.merino@tuebingen.mpg.de

You can find very useful advice in our publication: Protein denaturation at the air/water interface and how to prevent it: https://elifesciences.org/articles/42747 Davide Floris dafloris@biophys.mpg.de

One thing worth checking is to image a range of ice thicknesses in your existing cryo grids. If you are just imaging in the thinnest ice it may be that you have excluded your hexamer and are just seeing smaller complexes. In that case the hexamers may be hiding in thicker ice. This has happened to me in the past and I initially mistakenly concluded that large particles seen in negative stain were being replaced by subcomplexes when I imaged in cryo. On checking the thicker ice, I realized that this was not in fact the case. Ed Morris Ed.Morris@icr.ac.uk

If protein complexes are too small and you cannot get enough contrast with graphene oxide or in thicker ice then you can try a strepavidine monolayer. Jay Rai jrai@fsu.edu

You will see from the many responses that you are not alone in experiencing this problem of a specimen looking significantly different in negative stain vs cryoEM! Unfortunately, at present, there is no real "silver bullet" to tackle this, and the only way forward is to test a whole load of different variables until you find the thing that works for your protein. There have been some excellent suggestions so far, but I want to expand on these. (Caveat- only start doing these if you are confident in the quality of your prep-, you say negative stain looks nice and I expect you have biochemistry as back up (SEC (MALLS), native gels, native MS, CD, functional assays, etc.).

1) The first thing to check is that your particle is definitely dissociating and that you don't have intact particles. There could be other reasons why you aren't seeing particles in the holes of your grids. The particles could strongly partition to the carbon support film. In these cases, it can sometimes be hard to distinguish particles from the amorphous carbon. If the particles are there, but on the carbon, try altering glow discharge/plasma treatment, change to a different support material (like ultrAufoils), or investigate the use of a continuous film (GO or continuous carbon, depending on the size of your particle). As Ed pointed out, a second reason could be that the ice is so thin that you are physically excluding the particles from the holes. Looking for thicker ice will confirm this, but then smaller hole diameters may help to achieve more uniform thickness ice without the thinning in the middle that leads to particle exclusion from the holes. This is most common with larger specimens such as viruses.

2) If you are sure the particle is dissociating, as others have pointed out, the air/water interface (AWI) is potentially to blame. There is a lot of excellent literature on why this is the case (including the excellent

paper Davide pointed out) but here is a quick summary of things you can try, in no particular order;

A) Move the particle away from the AWI by sequestering it at the support water interface. This includes easy-to-try options like continuous carbon (if you have a particle where you have sufficient contrast to still align with the additional noise from the carbon), graphene oxide (or other engineered graphene supports https://www.pnas.org/content/116/24/11718) or "affinity grids" including the streptavidin monolayers that were mentioned, although these might involve some additional work with your protein.

B) Change the properties of the AWI by using a surfactant. Unfortunately, a huge range exists, and it seems that one detergent or concentration isn't the magic formula that works for all proteins, so there may be a lot of variables for you to try here. Common ones to try are DDM, LMNG, CHAPSO, and NP40. Stick to sub CMC concentrations for detergents.

C) Try a different device. The shear forces induced by filter paper based blotting might be your culprit (https://www.cell.com/biophysj/fulltext/S0006-3495(19)34402-9). Spotiton/Chameleon/Back-It-Up/Shake-it-off, CryoWriter and time-resolved cryoEM devices all avoid using filter paper. But interactions with the AWI occur within milliseconds so even with the fastest of these you won't outrun AWI interactions and the potential effects (see the preprint in D).

D) If you can, apply a higher concentration. If the protein itself is denaturing at the AWI, it will act as a surfactant which may prevent further denaturation. Saturating the AWI may also have positive effects on the angular distribution of particles that remain in the bulk of the thin film (away from the AWI): https://www.biorxiv.org/content/10.1101/2020.05.14.095372v1

E) Approach the problem from a biochemical perspective and crosslink the sample.

We have published a bit of a review about different variables that can be changed to alter particle distribution: https://journals.iucr.org/d/issues/2018/06/00/ic5104/index.html Rebecca Thompson R.F.Thompson@leeds.ac.uk

We had a case with an amphipathic protein in detergents that became suitable for cryo imaging only when being positioned tens of nanometers above the ChemiC films we developed (PMID: 24457027 and PMID: 30456382). The same method is suitable for modifications of graphene films, and it avoids ample biomass such as PEGs, DNA origamis, protein-binders, or other polymers, etc. It might be able to overcome the problems you encountered and help keep ice thin. Qiu-Xing Jiang qxjiang@ufl.edu

The pH of uranyl acetate (UA) is ~4. The common buffer for a protein sample is pH ~6–8. Depending on the staining method, the lower pH can be buffered. Moreover, UA acts as a fixative, preserving many protein-protein interactions on a millisecond time scale. Thus, can you give us some explanation of the UA staining affecting the structure? The overall structure or secondary structure? Zhongwu Zhou noonzhou@gmail.com

"The pH of UA is ~4. The common buffer for a protein sample is pH ~6–8." This provokes an answer. This is questionable, IMO many proteins investigated are cytosolic proteins (yes, not all, I know). The pH inside the cytoplasm is at or around 6 or 6.5, but not higher than 7. Thus, the TRIS-buffered solutions with a pH of 7.4 or higher as often used are not physiological (at all). Whether this is bad for the protein or not I do not know. BUT, keep this in mind.

"Depending on the staining method, the lower pH can be buffered". It is questionable whether a UA solution with a buffer at pH

>5 (or 6 or 7) is "stable"? I would not want to try this out. What happens with the UO_2 ion (which in UAc solutions are complexed by acetate ions)? Often, it precipitates. Note that UO_2 ions are less / barely soluble in the presence of carbonate (CO_3 (2-)), and phosphate is even worse. Precipitation.

"Moreover, uranyl acetate acts as a fixative." It is not a chemical fixative like formaldehyde or glutaraldehyde. If you use the term 'fixative', you should say what you mean. It is kind of bringing the protein close to its iso-electric point (pH 4.5 +/-), where the protein is at its point of "lowest solubility". Is this what you mean with "fixative"? "Preserving many protein-protein interactions on a millisecond time scale." I would say - arresting or reducing the protein's flexibility and functionality so they get out of their physiological status.

"Thus, can you give us some explanation of UA staining affecting the structure? the overall structure or secondary structure?" By getting close to the iso-electric point, I do not predict how much the tertiary or secondary structure is altered. Is it altered?? It is just the ionic groups in the side chains, which become less charged, less polar. Is this denaturation? Not necessarily. Just my 2 Euro-cents. Reinhard Rachel reinhard.rachel@biologie.uni-regensburg.de

I think the demonstration of UA being a "fixative" (on the millisecond timescale) comes from our 2003 JSB paper https://pubmed.ncbi.nlm.nih.gov/12576019. No, it is not a chemical crosslinker, but it stabilizes protein structure in some way, which is still not understood. As far as we could determine it was not simply due to a reduction in pH. Roger Craig Roger.Craig@umassmed.edu

Peak Masking in EDS+SEM
Microscopy Listserver

We are having a discussion in the lab about a possible caveat/issue using EDS to element map a mineral sample in SEM. Without disclosing too much about our project, I can say that the matrix is an alumosilicate which at one location is highly enriched in an element X. When an element mapping (including line scan) is performed at this location, all we can see in EDS is element X; Al and Si almost disappeared. Now the question is whether there really is no Al and Si at this location or whether they are present but cannot be detected because X is so enriched that it somehow hides Al and Si. My opinion is that as long as the sensor is not saturated (deadtime >0), Al and Si should be present in the spectrum, even though one may need to increase the dwell time significantly (except if X overlaps Si and/or Al, but both is impossible I guess). My colleague reasoned using a "constant sum", which I don't know of, to explain why X can mask Si and Al. I need an expert opinion on this matter. If X can really mask Si and Al in EDS, would WDS offer a solution? Thank you in advance. Best regards, **Stephane Nizets nizets2@yahoo.com**

Unless the peak from element X overlaps the Al and Si peaks, I would say that you should still see the matrix signal when the beam is next to the precipitate. Depending on the geometry of your system, if the generated X-rays pass through the precipitate on their way to the detector, there may be some significant absorption of Al and Si on one side of the precipitate. If the precipitate is large enough, you have a reasonable dead time, and it is not showing Al and Si, then I would say that those elements aren't in the phase. I'm not sure what your colleague means by "constant sum". The only thing I can think of is that, visually, if peak X grows quickly, the Al and Si peaks shrink in comparison. Henk Colijn colijn.1@osu.edu

It's always hard to say much without full information. It would be nice to know what element X is and the rest of the composition. It might

also help to know the EDS model involved and the instrumental conditions. Mapping is not all that sensitive to changes in composition. Linescan is a little better, but spectra are the most sensitive to seeing small differences. I use mapping to reveal gross changes in chemistry. The noise per pixel obscures small changes, especially the way that I see lots of maps done.

You mention difficulties with mapping, and you make a quick reference to linescan, but you did not say anything about regular spectra. Did you also collect them? That would provide a lot of information and might answer the questions. You did indicate if you mapped with spectral imaging where all the spectrum is available at all points. If it does, then you could reconstruct the spectra for the phases and not go back and collect spectra specifically for the phases. I would really want to see the spectra. Is there something markedly different between the spectra? Are the Al and Si still present at a low level? Suppose you did quant on the phases without normalization. Would the two areas give similar totals? If they did not, then something else is going on. I appreciate Henk's comments in another reply. I also don't know what your colleague means by "constant sum." You have also said nothing about oxygen. I think of two strange phenomena that may have caused problems. I don't know if they might be at all related to your situation. First, I remember someone giving a nice demonstration of the effect of count rate. I don't remember who it was, it has been many years, it might have been Chuck Fiore. The task was to map a copper TEM grid. They setup the SEM for brightness and count rate. They knew they had to turn up the beam current from what was normally used for imaging so they setup for 30% deadtime using the entire field of view, maybe more. When they collected the map, they found Cu intensity in the holes of the grid and practically nothing on the bars of the grid. I should have said "Cu" intensity. It was the old school approach of mapping a region of interest without any background correction. When they were mapping the holes, they got some "Cu" intensity from the bremsstrahlung background. When they mapped the bars, the count rate shot up many times higher. If their input count rate was set for 20 kcps over the entire grid, it probably jumped to 200 kcps on the bars and that effectively flooded the amplifier and shut it down so very few counts got out. So, the holes had "Cu" and the grid bars had none. Now I tried replicating this experience on an old Oxford ISIS, but I could not do it, *per se*. The ISIS corrected pixel dwell time for deadtime. So, if the deadtime went from 30 to 95%, my nominal dwell time of 10 ms went from an actual dwell time of 14 ms to 200 ms (10ms*100%/(100%−95%). It gave the proper result, but the scanning was quite strange as the beam slowed to a crawl over the bars. Second, I once tried analyzing a rare-earth phosphor that was severely cathodoluminescent. (Why should that be a surprise?) When the beam hit the phosphor, the count rate went through the roof. That meant deadtime went to 100%. That shut down all output on a real-time basis. Even with deadtime correction, the analysis was impossible. In Oxford's defense, I cannot remember if this was their original detector or a replacement detector from Gresham. I do know that our current light-element detector is still subject to interference from light photons whether from the room or the IR camera in the SEM or from cathodoluminescence. Not knowing what element X is, I don't know if this might be relevant. Warren Straszheim esaia@iastate.edu

Thin Dense Slice Burning a Hole in Carbon Films
Microscopy Listserver

We are trying to do EBSD (in an SEM) on a dense thin slice (produced by FIB). The thin slice is mounted on a carbon film, on a copper TEM grid. The problem is that each time we load a FIB-produced slice mounted on a carbon film-coated copper grid, the slice disappears within a short time of the beam going onto it. The hole remaining in the carbon film is the same shape as the original slice, so I suspect that the slice is heating up and burning a hole in the film. The remainder of the film remains intact. Any thoughts on how to mitigate this? Thanks. ***Allan Mitchell allan.mitchell@otago.ac.nz***

Some details on the nature of the sample could have been helpful, but if I had to guess: Try doing FIB deposition on a SiN window instead of a C grid. Prior to depositing the sample, coat the SiN window with C evaporation and make sure to provide reliable connection to ground for charge dissipation. If the "dense slice" isn't conductive it may be blowing up by ESD due to charging by high electron beam current. If so, you could try depositing a couple of nm of C on the side facing away from the EBSD camera and lowering the acceleration voltage. Valery Ray vray@partbeamsystech.com

Is there any chance that your sample is hydrated or is reacting under the beam to release oxygen? Try another sample material like silicon and see if it is still a problem. If it is, you might have a vacuum problem in your microscope due to a water or air leak. If it is, it's reacting with the film and beam. I would try silicon nitride films. Scott Walck s.walck@comcast.net

I would suggest using something like a SiN window grid rather than a carbon grid. They should be much more resistant to heating related problems. Nick Seaton seato008@umn.edu

Have you considered coating the whole sample? We use 1 nm of iridium for EBSD, which works for most samples including fragile biological specimens. I don't know how thick your "dense thin slice" is? Bil Schneider wfschneider@wisc.edu

— Mт

Crossword Puzzle Answers
See puzzle on page 64.

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