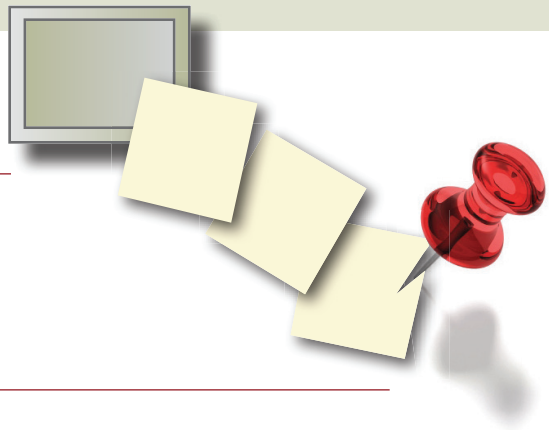
NetNotes



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

COVID-19 and Cryo-Electron Microscopy

3DEM Listserver

Dear all, I apologize for a naive question. I'm not an expert on viruses. However, I would like to know if there is anything we can do for the researchers working on COVID-19. This could be, for example, providing access to microscopes, imaging samples under safe conditions, analyzing images, sharing data, and answering questions. A negative answer will also be helpful, thus we could concentrate on other things. ***Daniel Levy Daniel.Levy@curie.fr***

It is not a naive question; it is a compelling one. If our facilities will be shut down for months, we should think of ways to make them useful to the research community combatting COVID-19. Perhaps a possibility is to coordinate with virologists who have access to recombinant viral proteins that may be imaged by cryo-EM. Alfredo De Biasio adb43@leicester.ac.uk

I think a lot of us are wondering how to be useful during this crisis; a couple of researchers from our institute (and beyond) have set up a COVID-19 crowd-fighting platform: crowdfightcovid19.org; if you are interested in joining us, please find information and contacts below. Celia Plisson celiapliss@yahoo.fr

We are a platform aiming to redirect scientific resources towards the fight against COVID-19 (crowdfightcovid19.org). This platform has two goals: 1) For scientists at all levels of their career, even if they work in fields completely unrelated to COVID-19, simply fill out a form so that we can contact them if we need their help. 2) Researchers already working on COVID-19 ask us to perform any task for them, for free. These tasks can include labor intensive tasks (annotating data, analyzing images manually, etc.), answering questions, or setting up new techniques. Everything is coordinated by scientists highly skilled in the field of interest, so the input we need from them will be kept to a minimum. Please, have a look to our platform (crowdfightcovid19.org) and sign up if you want! Crowdfight COVID-19 Crowdfightcovid19+contact@gmail.com

Another consideration might be to make your computational resources available for data analysis related to COVID-19. We have made our GPU cluster available to our bioinformaticians for base-calling nanopore sequence data. Centres engaged in ramping up their sequencing throughput may not have the computational resources to match their data output. David Bhella david.bhella@glasgow.ac.uk

Certainly, we should make clear that our infrastructure is open and ready for the challenge. Indeed, let me reiterate something you already know. Instruct (the European Research Infrastructure for Structural Biology) is offering its microscopy platforms in a priority manner for COVID-19 projects. Please, look at the Instruct Catalogue website. Unfortunately, the situation is such that none of these platforms are "operating as usual", but if you feel any of them may be of help in your COVID-19 research, please contact Instruct coordination at admin@instruct-eric.eu. Jose Maria Carazo carazo@cnb.csic.es

This is to announce that we are opening our cryo-EM facilities for COVID-19 projects at the Centre for Integrative Biology (http://www.igbmc.fr/grandesstructures/cbi/), Illkirch/Strasbourg, France, which hosts the French and European Infrastructures for Integrated Structural Biology, FRISBI (http://frisbi.eu/), Instruct-ERIC https://www.structuralbiology.eu/) and iNext-Discovery (https://instruct-eric.eu/news/eu-inext-discovery-grant-provides-technologies-for-key-research-in-structural-biology/). Research can include, for example, work on COVID-19 related enzyme targets, genome-free capsids, membrane receptor complexes, antibody complexes, etc. (i.e., non-infectious samples). Cryo-EM samples (on grids) should be sent in with dewars as no on-site visits by users are possible. We also offer freezing and screening of grids for purified samples. Apply please see below. Bruno Klaholz klaholz@igbmc.fr

COVID-19 and Core Microscopy Facilities

Confocal Microscopy Listserver

GermanBioImaging (GerBI-GMB, Society for Microscopy and Image Analysis) has improved and updated the "Recommendations for Operating Imaging Core Facilities in a research environment during the SARS-CoV-2 pandemic". This document is now finalized and endorsed by DGE, the German Society for Electron Microscopy; DGZ, the German Society for Cell Biology; and DGfZ, the German Society for Cytometry). More information is available at https://www.gerbi-gmb.de/Corona *or by direct download of the PDF version at* https://www.gerbi-gmb.de/sites/default/files/2020-04/GerBI-GMB_Corona_Recomm_2020-01-04.pdf. *In parallel I have asked the big four microscope companies (Nikon, Leica, Olympus and Zeiss) for recommendations on cleaning microscopes in Corona times and compatibility of cleaning procedures and fluids with microscope parts. There was fast response from all of them indicating that they are or will work on it. Leica has a webpage:* https://www.leica-microsystems.com/science-lab/how-to-sanitize-a-microscope ***Roland Nitschke roland.nitschke@uni-freiburg.de***

Thank you for all these great resources! I was wondering if you or anyone has put some thought on how to train users on the usage of microscopes? When labs start to reopen (at least where they are closed) we will probably have to follow some rules, like staying 6 feet away from each other and, the one-on-one interaction at the microscope might not be an option for some time. Cedric Espenel espenel@stanford.edu

Writing from NYC, I'm very interested in prudent and science-based best practices for re-opening our core in the months ahead. We normally do a few trainings every week, but one-on-one training involves way too much back-and-forth to maintain proper distance and sanitation. I also think that training someone remotely (me at home, them at the microscope) is impossible. So, I am wondering if we should begin to offer something I've always avoided—doing imaging as a service. The main barrier to this has been the need for constant user input on "good" areas of interest and "good" images. Perhaps we could apply our newfound videoconferencing expertise to bring the *user* into the room remotely, so they could see the computer screen and tell us if we are choosing the right area and capturing the right detail. Has anyone tried this? Theresa Swayne tcs6@cumc.columbia.edu

I have been thinking along exactly the same lines just across the city from you. We do not normally perform service work, but these are special circumstances. For the users working on COVID-19 who are already trained, we are writing out practical guides for each instrument and we have installed Teamviewer on the instruments so that the staff can provide remote assistance via the VPN. We are all having to work this out as we go, and it is great to see everybody's ideas popping up on the listserver and elsewhere. I am immensely grateful for the quick and proactive work German BioImaging did in coming out with their guidelines - those are super helpful. As long as we stick to the rule of only one person per microscope room, and all of the recommended cleaning precautions (including arriving with pre-wiped and non-hazardous samples!), I think we should be able to get on with a fair bit of COVID research. Training, however, is undoubtedly going to bring new challenges. I think that we will need to use a combination of approaches. For certain of our systems, such as our Zeiss CellDiscoverer 7, remote training WILL actually be possible, using a combination of written guidelines and Teamviewer sessions via the VPN. For others, we may need to make brief videos showing the different hardware components, and the procedures for turning on, shutting down, and taking slides on and off. etc. - but once within the software, again, written guides and Teamviewer sessions might suffice. Finally, I will confess that I would feel safer myself if I knew that I am the only person ever using a certain microscope - then less stringent cleaning would be required, etc. So I am considering, for example, having the trained users using the confocals and the CD7, while I (and staff members, once they are back at work) might each be in charge of one more specialized microscope (e.g., the super-resolution systems), and at least temporarily, the only user on that system. Alison North northa@mail.rockefeller.edu

I have put thought into how to train users on the usage of microscopes. I do not see how to train users without a give-and-take at the instruments with up-close demonstrations and observations, passing the equipment back and forth, etc. It's the assignment we had in seventh grade to write a protocol on how to make a peanut butter and jelly sandwich; there was always an essential detail another classmate needed that had been left out, and in the end, without extensive prior knowledge, the protocol was insufficient. And in this case, to extend the metaphor, the microscope is a sandwich making machine, but some people need Nutella and others need BLT and others want a Reuben, but they have brought thick rye bread with seeds instead of the #1.5 thin sliced seedless rye required by the instrument. Also, the instruments have been squeezed into the smallest spaces possible with HVAC of varying behaviors. In setting up new safety protocols, we should not forget that science is based in empirical study as well as hypothesis. We are currently in a phase where hypotheses are running wild without much evidence. We need to test these hypotheses. A lot of good ideas have been floated here regarding cleaning and isolation, but we don't really know to what extent they are effective or even needed (maybe we need more; maybe we don't need them). If we're going to make dramatic changes, let's have them based in science, like the rigorous sample preps and image quality that we espouse. Michael Cammer michael.cammer@med.nyu.edu

I agree with you. We are just starting to discuss what a return to our facility might look like. I am currently taking my cue from the German BioImaging group and microscope cleaning protocols we are discussing but also from the clinical teachers at our institution. In my opinion, if they only train when wearing PPE (learners also wearing PPE), then we should do the same. I can't imagine teaching someone how to use a microscope remotely at this stage as I simply haven't had enough practice to be able to do it well and it wouldn't be safe for the microscopes. I often have to give advice on specimen preparation as well and part of training involves building that relationship with the user. I am comfortable with authorized users (already trained) using our systems when we first return with occasional support from us if needed (we can keep the 2-meter social distance) but training new users requires a lot of thought. We are potential conduits of transmission, so we need to remember that we are not only protecting ourselves but others. Jacqui Ross jacqui.ross@auckland.ac.nz

I know that some cores are currently wrapping eyepieces in plastic wrap to minimize COVID-19 transmission, but this may be cumbersome and hard to keep in useful shape for users, especially during the eventual ramp back up when user flow is increasing. I wonder what the community thinks about having people wear safety glasses at the scope instead? Most oculars can be adjusted to accommodate glasses and safety glasses could easily be disinfected by the user. Thoughts? I have not had the opportunity to try it myself. Ben Abrams babrams@ucsc.edu

I have been wondering the same, but how would you adjust the oculars? I didn't get it to work properly (especially if you have to look for suitable areas for quite some time), so I dismissed this idea. Angela Kurz a.kurz@centenary.org.au

Thanks everyone for your replies to this thread. Angela, I did try this over the weekend and found that it was totally workable to wear standard safety glasses when viewing a sample through the eyepieces. I had to remove the rubber cups, but after that it seemed fine. I have mostly Zeiss and Leica scopes and tried it on a few different scopes and with two different models of eye wear. Neither were the splash-resistant kind used for chem labs. Ben Abrams babrams@ucsc.edu

I wear prescription glasses when I work at the scope. I do not take them off, but I do take the eye cups off the oculars. This works with all brands of microscopes. I used to need new lenses every 6 months or so, but in recent years there are new extra hard scratch resistant lenses that I have found are incredibly resilient. Also, they have great UV protection, as evident by how white the skin around my eyes remains even when I get tanned elsewhere on my face, and anti-reflectance coating that truly works. If you wear glasses, I recommend shelling out the money for premium lenses. And they may double as protective eyewear. Michael Cammer michael.cammer@med.nyu.edu

An alternative workaround would be to supply plastic wrap to cover the oculars. Each user will need to replace it. Further recommendations can also be found on the GerBI website: https://www.gerbi-gmb.de/Corona Gabriel Krens gabriel.krens@ist.ac.at

We have removed the eye cups, insist our users wipe the oculars with 80% (v/v) ethanol on a Kimwipe before and after each use, users

must wear safety glasses, gloves and a clean gown, and they must clean the computer mouse, keyboard, and microscope knobs with 80% (v/v) ethanol wipes before and after each use. Training is limited to "essential" projects only. Trouble shooting and training is achieved by attaching a second, remote computer mouse. The Logitech IR mouse with long battery life is good for this. Instructors can then stand quite a long way behind the trainee. The instructor can point to any part of the screen by operating the mouse on a bench or even their leg. In addition, knobs on the microscope can be pointed to using a cheap laser pointer. As my eyes aren't what they use to be, when selecting various options in pull down menus, etc., I will ask the trainee to confirm the option I'm selecting, making jokes that I'm half blind. The humor, and the process of asking the trainee to confirm the software selections, actually seems to aid training. When things return to normal, I think we'll continue with the second mouse training technique. So far comments from our clients have been highly complimentary regarding the new safety protocols. Stephen H. Cody stephenhcody@gmail.com

Core Facility Pricing

Confocal Microscopy Listserver

I'm wondering if any core facilities employ a sliding charge scale for instrument time based on cumulative hours, as in the more a lab uses confocal in a month the cheaper it gets per hour, or any other pricing scheme to alleviate cost for high-volume users (I know a lot of cores do a peak vs. non-peak hourly rate but I'm looking for alternatives to that). Any comments/recommendations regarding volume discounts or other ways to mitigate cost for major users are much appreciated. ***Esteban Fernandez g.esteban.fernandez@gmail.com***

Our core is required to charge all users the same price. We do have one microscope with a lower price for nights, weekends, and holidays (for fully trained users). All of this is easy to do with iLabs. We do informally (since I don't know of a trivial way to do this in iLabs) allow some major user labs to schedule further ahead of time than other user labs. In fact, I encourage the major users/labs to reserve time several weeks in advance, and if they want to routinely book (say) every Wednesday, 10am-5pm, great. Both of our main microscopes—Leica SP8 and Olympus FV3000RS—from mid-January until Coronavirus induced work stoppage—had gotten to the point of being booked almost full workdays. This pretty much meant no new users since we have a policy of training on the confocals generally two 2-hour sessions during the workday. No 2-hour slots available=no new users. I anticipate that when we do get back to work, the previous regular user base will pretty much book up all available time during workdays, so could be a while before I have to deal with social distancing vis-à-vis training users. George McNamara geomcnamara@earthlink.net

We apply a discount for time-lapse imaging over 4 hours for all systems, e.g., the first 4 hours are the standard charge and then it's reduced to 2/3 charge rate. The reduced charge for time-lapse studies is to try to make this more affordable as we do want to encourage people to do live cell/tissue imaging. We have also offered one system (a slide-scanner) at a reduced rate for after-hours usage to try to spread out the demand. Jacqui Ross jacqui.ross@auckland.ac.nz

Yes, for many years we have been using a "sliding scale" scheme in the form of volume usage discounts for our LSM510 and LSM780 that uses an hourly, prime time, full day use and yearly volume use. With these schemes in place major users may pay up to 8 times lower average fee per hr than occasional users (though total is fixed per year). Major users also have priority scheduling. Recently the power user discount was scaled down to a quarter (our billing cycle) because of charging restrictions imposed by our accounting (no volume prepay is allowed so power users are charged a regular fee until the quarterly limit is reached, after that usage is "free" until the end of the quarter). This approach allows the PI more uniform distribution of payments throughout whole year. This scheme makes life and budgeting easier for major users (currently we have two of them) but may reduce Core revenue. Arvydas Matiukas matiukaa@upstate.edu

Many cores are bound by grants or their institution to charge all users the same price. However (depending on what sort of agreements you have to work with) you can finesse rates to accommodate different kinds of use. In my last role I set one rate for assisted use, a lower rate for independent use (after passing a certification), and a night and weekend rate. The night and weekend rate was great for high-volume users and time-lapse studies, and it helped the core by shifting bigger jobs to a time slot that wouldn't prevent others users from working. Timothy Feinstein tnf8@pitt.edu

I was told by a consultant that was reviewing our policies in order to adhere to NIH, NCI as well as other funding bodies, that it is not appropriate to have differential pricing structures because it favors larger labs with more funding. A small lab with a small amount of funds will inherently do less imaging and will have to pay a higher price for it. Conversely, the labs with the most people and funding will use the systems more and will receive the biggest discount. This seems unfair. We do not have a decreased rate during off hours. In my opinion this encourages users to use systems late at night or on the weekends. When a user operates the systems late at night without any guidance the chances for misuse of the system increases. This relates to care of the system, laser safety and even the fidelity of the imaging parameters (and yes, I think we play some role in ensuring that grad students and post-docs are acquiring data that is ethical and quantifiable). For this same reason we do not charge for assistance. We want the user to ask for help rather than avoid it. We do have discounted rates for long-term imaging (>24 hours) for live cell imaging on the confocal and live cell imaging systems. Brian Armstrong barmstrong@coh.org

We have fee structures whereby work in the evenings or weekends may be less than peak hours. These fees must be applied equally to all users as per government mandates. Keep good records; you may be audited. There are discounts for researchers in certain programs, such as the cancer center or liver center users, but I believe the individual users are getting the discounts because the difference is made up by the programs paying. (In a previous position, some years the core collected more from the grants than the discount to users, so this was a good deal for the core.) I don't know the details of how this works where I am now (very glad there are finance people who handle this), but again, records are being kept (in iLab) to conform to government regulations. Michael Cammer michael.cammer@med.nyu.edu

When people run 24-hour experiments on our confocal instruments, the most that they will get charged in any 24-hour period is for 16 hours. This requires a bit of manual tallying when billing time comes, but it has been manageable so far and helps to facilitate around-the-clock use of our instruments. We've set up "per hour" and "per day" rates for this. We have been able to justify the discount based on lower cost and wear and tear on the instruments due to fewer sample changes during long imaging runs. Hope this helps. Ben Abrams babrams@ucsc.edu

Thanks for sharing everyone! It's great to get some perspective from hearing what others do. This will help us devise a new pricing

structure once this is all over and we get back in the lab. Esteban Fernandez g.esteban.fernandez@gmail.com

Calibration with Microspheres

Confocal Microscopy Listserver

We did some extensive intensity calibration experiments with different intensity green and red microspheres. The green microspheres were 3.7% and 35% relative intensities with an intensity ratio of 10.6. We measured the intensity ratio with lots of different microscopes and lots of different lenses and very consistently got a ratio of 8. I can't seem to figure this out at all. It means the bright beads must be a bit dimmer than expected or the dim beads a bit brighter than expected. The calibration would have been done by ThermoFisher—I would guess they do this by flow cytometry. Maybe it could just be that microscopy measures the intensities more accurately? Does flow just get one data point per sphere? Maybe the bright beads bleach relatively more than the dim ones when you are imaging on a CLSM? Any ideas are welcome! ***Claire Brown claire.brown@mcgill.ca***

Just curious - were the measurements done in water? Scott Henderson schenderson@scripps.edu

Perhaps they used a different emission filter? Michael Model mmodel@kent.edu

There are a number of possible ways to measure bead fluorescence, it would be helpful if you described your pipeline in detail. Are you segmenting beads by intensity and summing up the total fluorescence per bead? Or taking the average? Are you performing background subtraction? As Patrick inquired, are the beads the same size? The likeliest explanation, given that you're consistently underestimating the ratio between them is that there is a fixed background in the image. For example, if the dim beads represent 100 counts absolute and the bright beads 1060 for a ratio exactly of 10.6, a background of 50 counts would give you an apparent ratio of 8 instead. To correct for this, you can either subtract the background or do a 3-point calibration with 0-intensity ROI's of the same size as your beads are randomly distributed in the image background. Pavak Shah pavak@ucla.edu

They were done in CyGel from Biostatus Ltd. I guess there could be some quenching from the medium. The imaging was done on over 40 different microscopes with different magnifications, immersion medium and NA and instrument settings, which is why we used the intensity ratio. The dim and bright beads were in the same sample though. They are the same size, but perhaps the brighter ones could heat up more? Not sure how much heating you might get with a green laser though. It could also be that flow obtains the max intensity at the middle of the sphere but in microscopy we measure the whole thing. However, I would think that any difference like that would disappear with the ratio. We did correct for background so that is not it. I guess we could also have the wrong lot information. We got them from a 3rd party not directly from the company. Claire Brown claire.brown@mcgill.ca

Size of a Photobleach Point

Confocal Microscopy Listserver

When using the Bleach Point feature in Leica confocal systems (I'm sure it also exists in the other brands), I don't know how to measure the surface of the bleached area. I figure out the wavelength of the laser beam and the numerical aperture of the lens affects the size of that area, so would it be correct to use the resolution formula to calculate it? Or am I mixing up things here? ***Xavier Sanjuan Samarra xavier.sanjuan@upf.edu***

I tend to use the PSF generator in ImageJ/Fiji (http://bigwww.epfl.ch/algorithms/psfgenerator/). You can take the full width at half maximal to indicate the bleach area. In *x*, *y*, and *z*. I tend to think of bleaching a volume rather than an area. I'm sure this isn't perfect, as the energy isn't equally spread within the PSF. If I'm teaching this, I use the plot profile on live view of a PSF, fix the axes ranges on the graph, and run through the z stack to show the energy distribution in different z slices. Or save a small (65×65×65 pixel) PSF with 50 or 100 nm *xyz* pixels and use ICY 3D viewer with a color map (LUT) like Parula. I always need to fix the metadata in ICY to the correct *xyz* dimensions. Dale Moulding d.moulding@ucl.ac.uk

For fastest photobleaching, it is generally best to use maximum power for as brief a time as possible. The more photons, the bigger the spot. Oxygen radicals and dye radicals diffuse short distances relative to PSF (nanometers, though activated tyramide radicals have a diffusion radius of ~100 nm and can be restrained by increasing viscosity or additives. Cytoplasm has a higher viscosity and density than water, so the radical diffusion radius might be suppressed in live cells that are [over]expressing catalase). A(nother) good PSF calculator is available free at SVI (https://svi.nl/NyquistCalculator). I liked this recent publication on the photophysics and photochemistry of fluorescence and generalization across the UV-Visible fluorophores: Aleksandr Barulin and Jerome Wenger, 2020 *J. Phys. Chem. Lett.* 2020, 11, 2027–35. George McNamara geomcnamara@earthlink.net

For quantitative analysis of recovery after bleaching a spot with a Gaussian laser beam (TEM00), the beam waist typically is used for the radius term. This is the width at which the normalized intensity of the beam drops to 1/e^2. To measure the intensity profile of the beam, you would take an image of the parked beam on a uniform, photostable sample, use ImageJ to get an intensity profile across it and fit a Gaussian to the profile. In principle, you could also do this from the inverted intensity profile of the bleached region in the first image acquired after bleaching, but if recovery is fast, this will be an underestimate of the "true" radius of the spot. The FWHM is approximated by 2.355×the standard deviation of the Gaussian and the waist is about 1.7×FWHM. The difference is important for quantitative analysis to obtain the transport characteristics of what you are bleaching because the area of the bleached region is significantly larger using beam waist than using FWHM. Kate Luby-Phelps kate.phelps@utsouthwestern.edu

Plexiglas may not scatter and absorb light the same as your biological samples, but with SP2 AOBS and SP5 AOBS systems we bleached spots inside fluorescent Plexiglas and then went back and imaged a Z-series of the bleached volumes to see focal plane and cones above and below. Two examples: https://www.flickr.com/photos/mcammer/2605562876/ and https://www.flickr.com/photos/mcammer/2608080259/. Plexiglas previously worked well as a model for cell cultures, but we are having an issue now (ok, in February and maybe again someday…) that as we focus deeper in drosophila embryos using a Bruker galvo miniscanner that there is a lot of scattering. At the surface of a zebrafish embryo we can target membranes specifically, but deeper in drosophila scatters too much for fine targeting. We have tried three different lenses and adjusting the collar of one empirically. This is at 405 and 470 nm. So spot size is very depth and sample dependent. Michael Cammer michael.cammer@med.nyu.edu

Thanks for all the feedback! I did not want this for a photobleaching experiment, we have been using a multiphoton laser at 830 nm to

heat nanoparticles within cells, but I guess this does not affect the calculations! Xavier Sanjuan Samarra xavier.sanjuan@upf.edu

2-Photon Use for Zebrafish Axon Ablation

Confocal Listserver

We are in process of acquiring a 2P microscope. One of the applications is to cut zebrafish axon while imaging. We failed to cut nerves with a demo system equipped with Spectra Physics Insight X3 DUAL (tunable from 680–1300 nm plus a fixed line at 1045 nm). The attempt was done with a 25× water dipping lens NA1.0 without coverslip on a 7-day-old fish at about 750 µm *deep. I have to say the demo test was done in a relative short time on a new system which we don't know much. My questions are: Have any of you done 2P ablation with young/adult fish nerve? If so what equipment, laser, and parameters did you use to cut? Have any used a water dipping lens without coverslip? Any opinions about the laser used for ablation, Spectra Physics vs Coherent? Thank you very much in advance.* ***Gang (Greg) Ning gxn7@psu.edu***

You don't mention what wavelength you were using. Have you tried using deuterium for dipping? It's less absorptive than water. Marc Reinig mreinig2@gmail.com

The laser wavelength was 780 nm. Gang (Greg) Ning gxn7@psu.edu

We've ablated cells in zebrafish with an Insight DS (the predecessor to the X3 with less power), a 20×1.0 water dipping objective, wavelengths 800–900 nm. It wasn't easy though. I know of other groups who have compared the X3 to the MaiTai HP and found the shorter pulse width (80 fs vs. up to 200 fs) improves ablation ability. Douglas Richardson ds.richardson@gmail.com

About 10 years ago I ablated focal lesions in spinal cord with an old Spectra Physics Tsunami, which is pretty similar to contemporary Ti:Saph lasers. I believe the wavelength I used was 900 nm, and I dispersion-optimized the system for approximately 200 fs pulse width and used 10's of mW on the sample. My dwell time was fairly long, I believe on the order of 50–250 microseconds. As Doug mentioned, shorter pulse widths will improve efficacy, so consider a pulse compressor. Craig Brideau craig.brideau@gmail.com

Commercial Response I want to direct you to a couple of publications in which the researchers used the Andor Micropoint laser system for axon ablation at 440 nm: doi:10.1242/dev.004267 and doi:10.1038/nn1803. We have an excellent photo-ablation system that has been very successful in similar experiments and can be fully automated. It can also be used in conjunction with any microscope system. Kalpana Iyengar k.iyengar@andor.com

While the MicroPoint is indeed a nice system, I think it will not be suitable for what Greg and his users are trying to achieve for two reasons: 1) Reaching over 100 µm depth (750 µm is what Greg mentioned) with a 440 nm laser to ablate an axon (and not everything above it) will be nigh impossible. NIR illumination and 2P ablation is required. 2) My understanding is that the MicroPoint will only allow for targeting of a spot using a camera image, acquired in the software controlling the MicroPoint. This would not allow a user to acquire an image with the two-photon microscope and load that image into the MicroPoint software. In general, I can mostly echo what Craig and Doug have said: Yes, it is possible (I have seen it done in a range of tissues). Water dipping should be fine. A wavelength longer than what you initially used should be more successful. Scan slowly—potentially just park the beam on the axon. Repeated fast scanning will not cause the same damage as a single slow scan—usually we want to do the former to avoid damage, in this case you want the latter to cause it. And finally, yes, shorter pulses will be better. But keep in mind you will need to compress the pulses to the shortest possible duration under the objective. Ideally you would want to measure the pulse width under the objective with an autocorrelator (not cheap and takes a bit of time), or you can optimize the pulse duration while imaging using the wavelength you want to ablate with: change the GDD-compensation to get the brightest possible image (if you aren't doing that already). Coherent vs. Spectra: either should work fine. Christian Wilms christian.wilms@scientifica.uk.com

Hi Christian, Thank you for your response. You are correct in that I underestimated the need for penetration depth in this specific application. The MicroPoint has good power at 626 nm, however, we have not tested the depth specifications. If Greg or anyone else here is interested in investigating the depth requirements further, we are happy to help look into this. However, I want to clarify that your second point is not accurate. We have developed a Virtual Camera approach that is designed specifically to integrate with third-party systems like this. We are able to do exactly what you mention: acquire an image with the two-photon microscope and load that image into the MicroPoint software. Kalpana Iyengar k.iyengar@andor.com

A quick and dirty way to verify that you have near minimum pulse width is to grow a KDP crystal on a cover slip and use it to generate second harmonic at the focus of your objective. Just create a supersaturated solution of KDP powder in boiling water and put a drop on the coverslip. The crystals should start to form as the solution dries. You can also add a sprinkle of KDP powder to the drop to provide a seed and get better crystal formation. Adjust your pulse compressor to achieve maximum second harmonic production. This isn't perfect but will allow you to compensate for your microscope optics, which will be a far larger contributor to pulse spread than your sample. Craig Brideau craig.brideau@gmail.com

Hi, Craig. We normally used urea for this. It's easier to find in the lab. But a more general and pressing question is: Is the ablation indeed a two-photon process? If so, then I'd guess it would not be powerful enough to cause anything. Pure absorption will be much stronger, and pulse duration will not matter, as long as it's in the nanoseconds or less, to prevent heat dissipation. And as a note, I would be very surprised if any decent femtosecond laser wasn't able to cut an axon. One can "boil" the sample if not cautious enough, and some papers I've seen just park the laser on the cell for one second at 100 mW power… this has to work. And if it still does not work, making the cell a little bit more absorbent at the NIR wavelength will enhance the effect dramatically. I'm thinking of something like Indocyanine Green (though I have no idea how to get it into the cells, or at least close to the surface). Zdenek Svindrych zdedenn@gmail.com

Thanks for that correction, Kalpana! Last time I spoke to one of your colleagues in the UK headquarters was nearly a year ago. At the time I was told that function was not available and wasn't planned – so I am glad to hear that has changed. Thanks, Craig for the advice on using SHG. Christian Wilms christian.wilms@scientifica.uk.com

Carbon Coater

Microscopy Listserver

We are seeking to purchase a new carbon coater. Our center does EDS and EBSD and we would like to coat down to 5 nm of carbon. We

are looking at both carbon rod coaters as well as a carbon thread coater. I would appreciate input on these two types of coaters. ***Pat McCurdy pmccurdy@colostate.edu***

I have only done carbon rod and an old Gatan PECS ion deposition system. My qualitative observations are below, and I have no financial interest in coating materials companies. The PECS is slow, expensive to operate, and for our samples was limited to one at a time, but it worked well until we stopped maintaining it due to lack of use. We have a 10-year old EMS rod type turbo pumped carbon coater (not the one they currently sell). It works fine down to 3 nm on EBSD but is a bit trickier to get the desired thickness. Consistently sharpening the rod is key to getting a consistent thickness. Some of my geology users complain that they have to run two coating cycles since the sharpened rod will not get them to a 25 nm coat if they are going to microprobe. If you want a high-quality C coat high vacuum is a must. Greg Baty gbaty@pdx.ed

With due disclaimer that (a) I've been doing C coatings mostly for charge mitigation on FIB circuit edit samples, and (b) below are personal impressions and not a conclusion from any kind of comparative study: The best (perceived as smoothest, cleanest, and most uniform) carbon coatings I've seen were produced by Gatan's PECS system, using ion sputtering. I haven't operated PECS myself, but for me coatings made in it were perfect. Overall impression is that good cord and rod evaporation coatings typically come from turbo-pumped systems run by an operator with enough patience to wait for a full pump-down. I have been using the high-vacuum version of Safematic, and despite my initial skepticism I am very pleased with it. Automated exchange of evaporation cord is oh so convenient. No vested interest in Gatan/AMETEK or Safematic. Valery Ray vray@partbeamsystech.com

Great question for the ListServ. There are advantages for both rod and thread for carbon coating. There is also e-beam carbon and thermal evaporation which are more costly but can control a more precise and thinner layer. If you go with traditional rod or thread coating, then you should get a system with high vacuum (TMP) to have a finer grain size since you want a layer down to 5 nm. While a carbon rod can be more precise for very thin layers and finer grain size, some thread systems can be pulsed, so the coating occurs slower to produce good control of thin layers also. Thread systems are also a little easier to use since you do not have to sharpen and handle delicate carbon rods. A thread system would be good for new users that just want a quick conductive coating. Why compromise though? There are a couple of systems that can do both rod or thread, or rod capability can be added later as an upgrade. We offer such a coater: https://elementpi.com/sputter-coaters-carbon-evaporators/. Mike Toalson miketoalson@gmail.com

We have a Denton 502A Carbon Coater with a turbo molecular pump and Cressington thickness monitor. Using carbon rods, we deposit between 7–10 nm for CL and 20 nm for imaging, EDS, and EPMA. It has a cold finger so liquid nitrogen can be added to hasten a quick coat, otherwise an hour pump down gets us to 10^{-6} torr. The stage rotates as it is a line-of-site coater, 10 one-inch rounds or maybe 5 thin sections can go in at one pump-down. We do a ton of EBSD but rely on a Leica Ace600 coater to apply a 1 nm coat of Iridium to any EBSD sample. It is flawless on geological thin sections and never charges. Bill Schneider wfschneider@wisc.edu

Cleaning Nickel Shim of Magnetic and/or Glass Particles

Microscopy Listserver

I have a nickel shim destined for nanoimprint lithography, made by electroforming e-beam exposed photoresist. I don't have a proper cleanroom, but I've been trying to strip what seemed like residual ZEP e-beam resist and it has not been going so well. I've tried acetone, dichloromethane, n-methyl pyrrolidone, and 10% NaOH and sonicated with heat in both acetone and NaOH (at different times). The NaOH is the most recent attempt, and it seemed to show improvement under FIB imaging, but I also noticed what appeared to be redeposition. I can only imagine this is due to particulate in my solvents, dirty air as I blow dry the shim or carry it from my sonicator to my FIB desk, or maybe insoluble particles like glass or ferromagnetic dust which start to settle onto the sample as soon as the sonicator is turned off. Features are around 150 nm linewidth, high frequency and complex shaped. So, lots of small approximately 500 nm sized holes/crevices which I thought was just diffusion limited for the solvent to get into and do its work. But now I'm confused. Should I invest in some .45- and .22-micron syringe filters for all my fluid work? Should I tape a magnet to the outside of the beaker I've been sonicating in to try and collect such particles? What is a standard semiconductor lab method for cleaning magnetic particles from magnetic layers? How about the idea of insolubles? Or can someone recommend a solution that will etch glass but not nickel? ***Nathan McCorkle nmz787@gmail.com***

Did you try using a plasma cleaner for cleaning the surfaces and also a plasma cleaner like the Evactron at the FIB chamber to keep the specimen clean during scanning? If you can mount the specimen with the surface to be cleaned facing down to the bottom of the beaker you might get rid of deposits coming from above. Another way to try to clean the surfaces might be to plunge in liquid nitrogen or to use a vacuum chamber with the cleaning solution and pump to a level below sublimation. And sure: clean micro-filtered solutions would help. Nickel and magnetism: you could use a demagnetizer to decrease/erase the magnetism in the shim first. Stefan Diller diller@stefan-diller.com

To clean a surface of particulates I would use replicating tape. This is a cellulose acetate tape (non-adhesive) that you soften with acetone and press down onto the surface. Let it dry and peel it off. All the particulates should come with it. I've had better luck in removing particulates this way compared with ultrasonics, rinses, etc. I'm not sure if an adhesive tape will work but if you don't have replicating tape, you might try some of the tape with the "Post-it" type adhesive. It may take several applications to remove everything. Replicating tape is available from most of the EM supply houses. It comes in both a thick and thin form. Hendrik O. Colijn colijn.1@osu.edu

Particle Analysis

3DEM Listserver

I prepared cryo grids for single particle analysis for 600 kD protein-RNA complex. The particles are overcrowded. After dilution, the particles are not separated. When I diluted it further, holes became empty. Gel filtration makes a single peak, but on the grid they stick together. I have already tried glycerol, DMSO, amphipols, salt concentration 70–400 mM. None of them worked well. The buffer contains 150 mM NaCl, 10 mM HEPES pH8, 0.5 mM EDTA, 4 mM DTT. The sample had about 1mg/ml, blotted for 3 sec, force 0, 100% humidity, 22C on Ultraufoil R0.6/1.0 coated with graphene-oxide. The image was taken with a Tecnai12. The particles are difficult to get into the holes without graphene-oxide. Any expert suggestions will be helpful. ***Satoru Machida dbssator@nus.edu.sg***

You do not state the source of your protein-RNA complex - but most likely, under physiological conditions, it is an "intracellular" complex. Which cell – prokaryotic, i.e., bacterial, or archaeal? or eukaryotic? organelles of a eukaryote? This is important to know. The optimal temperature is also important to know. My suggestion is your buffer.

IF this complex is located inside the cytoplasm of any given cell, the optimal pH would be close to pH 7.0 (with quite some variations, depending on the physiology of the cell!). This means that the conc of H+ ions ideally might be 10 times higher than in the buffer you are using. Second: NaCl as the main salt used in your buffer (150 mM) is close to what is optimal for extracellular environments but NOT to intracellular conditions. I doubt that this is ideal for your complex under investigation. Inside cells, you have a balance of K+ and Na+ ions, with 3 to 10(0)×higher conc of K+ (!!). In addition, there is some Cl- found inside cells, but the dominant counterions are phosphate groups from nucleic acids and metabolites, and carboxylates (from metabolites). Can Cl- be used? They may, but if you fail, you have to think about this. Thus, I would use HEPES at pH 7; use the optimal temp for your complex before starting the freezing process (which organism at which "optimal" temp? this influences the real pH); and a balanced amount of lots of K+, little Na+, and counterions (you may start with Cl- salts, simply for ease of use; but later, you may think about phosphates and carbonates). Adding EDTA and DTT is common, but may not be physiological? However, it might help. EDTA substitutes for the fact that inside a cell there are many metabolites with similar "complexing" functions; DTT substitutes for the fact that inside a cell there are usually quite complex systems for keeping the Redox state in balance (i.e., reduced). This again depends on your type of cell. Reinhard Rachel reinhard.rachel@biologie.uni-regensburg.de

I would try Quantifoil carbon grids R2/4+ or R3/3+2 nm additional carbon film. Always check NS first and then go into cryo. Use 100% humidity and 4°C. Blot for only 2 or 4 seconds. If you succeed, then you can increase the sample concentration by a factor of 10 and freeze 1 or 2 sec R1.2/1.3 without carbon. Good luck. I know every grid looks different. Jorg Buerger buerger@molgen.mpg.de

Are you sure the particles are intact after freezing? If the protein complex dissociates on the air-water interface, it might look like poor particle spread. Also be aware that textbook-like particle spread is desirable, but by no means a necessary requirement to obtain high-resolution reconstructions. If you can clearly see the particles, try collecting a small dataset on a better microscope. You might be surprised.

Other ideas to improve your sample: Crosslinking (this might not be ideal for nucleic acid—protein complexes because the crosslinker can modify lysine residues in the DNA binding interface, so check the crosslinked sample with an EMSA. Crosslinking has the advantage of stabilizing the complex through intermolecular crosslinks and passivating the surface through mono-crosslinks) and detergents (when detergents are used increase the protein concentration, aim for around 2 mg/mL at least). Matthias Vorlaender matthias.vorlaender@embl.de

Orientation Issue with Membrane Protein Structure

3DEM Listserver

I am trying to solve the structure of a tetrameric membrane protein complex with the protein embedded in detergent micelle (0.05% GDN) and a soluble accessory protein attached to it. Following 2D classification, while the side views and oblique views are easily visible, the top and bottom views are few (~1–2%) and further diminished in subsequent rounds of 2D classification. While we have tried different grid types to overcome the orientation problem at the sample level, I was wondering if there are certain tweaks we can make to the analysis parameters (particle picking, box size, mask, contrast transfer function, etc.) to enhance the signal of a protein embedded within a micelle in the current data set. We are using Relion 3.1 for SPA. Any suggestions to salvage this set of data will be very helpful. ***Saumya Bajaj saumya.bajaj@ntu.edu.sg***

Assuming this is a symmetric membrane protein with at least 3-fold rotational symmetry, you don't need the top and bottom views to fully sample Fourier space and arrive at a high-quality reconstruction. Side-views of a rotationally symmetric particle are sufficient, and the reconstruction will be complete. Dmitry Lyumkis dlyumkis@salk.edu

Why do you say that the symmetry has to be at least C3? Philip Koeck koeck@kth.se

I am also confused by this. Shouldn't a tomographic series (180 degrees worth of side views) do it for a C1 particle? Basil Greber basil-greber@gmx.net

If it is a two-fold rotationally symmetric object, adhered to the air-water interface along a single side view, then a projection along, e.g., phi=0°/theta=90° samples the same Fourier plane as the second projection along phi=180°/theta=90°. The reconstruction will behave in a manner that is identical to one that is composed of exclusively top views of a rotationally symmetric object (or, more simply, an asymmetric particle with one preferential orientation, normalizing for the number of asymmetric units). Basically, you will end up hyper-sampling around one plane in both cases, except that the planes will lie along distinct axes of the transform. Both are bad cases and, in the absence of other views, will lead to a bad reconstruction. To be clear: this is assuming that there is only one preferential orientation along the side view of a two-fold rotationally symmetric particle, and not more. If you have 3-fold rotational symmetry, and the sample is adhered to its side view, projections separated by phi=120° spacing will lead to sampling of Fourier planes separated by phi=60° (due to symmetry). Effectively, you add two additional planes. Assuming you have enough particles, and there is a bit of spread in the phi angle, in most practical cases, the reconstruction of a 3-fold rotationally symmetric object will be complete (or nearly so). The higher the rotational symmetry, obviously the better. To answer the original question, adding top views for your membrane protein will be negligible in the reconstruction. Looks like you should already get a very nice map from the current data. Give it a try. Dmitry Lyumkis dlyumkis@salk.edu

You are completely right. 180° of side views about a single axis samples 100% of 3D Fourier space with a C1 particle. Ed Morris ed.morris@icr.ac.uk

This is done all the time in helical reconstruction, to resolutions better than 3.0 angstroms. Having all projections of side views is a single-axis tilt series, which yields all information needed in the absence of any symmetry. Edward Egelman egelman@virginia.edu

To be honest, I'm a little surprised by this discussion, as it is part of most basic Cryo-EM intro courses. While Ed is correct for helices, of course, and while having all possible side views does, indeed, yield a complete data set, there is a problem in the case of single particle analysis. In helical reconstruction, once the symmetry is known, there is a relationship between linear position along the length of the helix and orientation about the helical axis. In the case of single particle analysis, with only pure "side views" (perpendicular to some axis), there is no information available to accurately determine the angle about the symmetry axis, since the only common line the projections share lies on the helical axis. While there are approaches to come up with reasonable results (for example the "sidewinder" program developed by Penczek) to achieve good

particle orientations requires at least having reasonably good tilted views to anchor the orientations. "Top" views are not required, of course, but if only pure side views are available you will definitely run into problems. You might get by with 10–20° tilted views, but it's best if you have a population that goes up to at least ~45°. Steven J. Ludtke sludtke@bcm.edu

I believe you are right if you can be sure that you've found a single perfect side view and the symmetry is at least C3. In that case I would say you can just symmetrize, as you describe, and get a rough 3D model without using either top or tilted views. Note: You are using a single side view! I don't see how a spread of different side views would help you though. You run into the problem that Steve pointed out. You don't know how the different side views are oriented with respect to each other unless you have at least one top or tilted view to lock the relative orientations. Philip Koeck koeck@kth.se

This is why single particle analysis is a local minimization dependent on the initial model used. The assigned orientations are based on this initial input and providing Fourier space is well-sampled and the input is within the correct local minimum, the angular assignment and calculated 3D structure will converge. Christopher Aylett chsaylett@gmail.com

Does it mean that we can reconstruct a 3D map of a membrane protein having C3 symmetry just from pure side views? Shashi Bhushan shashibhushan.tyagi@gmail.com

You certainly can if you have a good enough starting model to align your images and to assign the Euler angles. You can also do it with less symmetry, but the quality of the starting model will be more critical. Ed Morris ed.morris@icr.ac.uk

MT

Crossword Puzzle Answers

See puzzle on page 68.

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

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