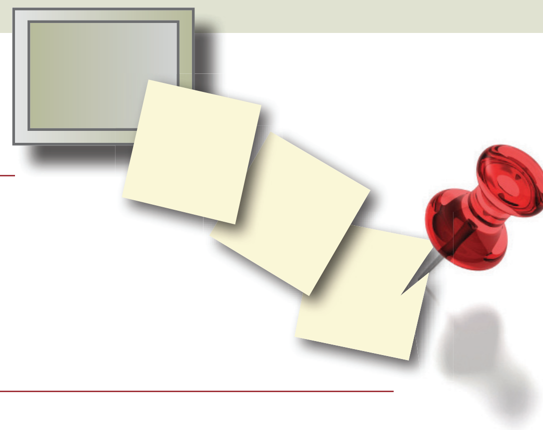

NetNotes

Edited by Bob Price
University of South Carolina School of Medicine

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Moving Scanning and Transmission Electron Microscopes

Microscopy Listserver

Dear Fellow Microscopists, we are currently planning to move our equipment, which includes 3 SEMs (JEOL 6500F, EVO 50, Supra 40), 2 TEMs (JEOL 1230, JEOL 2100), 2 XRD, 1 XCT (NIKON), 1 Confocal (Zeiss LSM 510), 2 AFMs (Bruker Dimension Icon, Bioscope), and other smaller optical microscopes and prep equipment to our new facility. We were asked to consider the possibility to move all of them ourselves. My questions to the group are 1. If we were to entertain this possibility, I would like your opinion on mistakes that we can or need avoid. 2. Some advice if there is a low-cost alternative to getting the instruments moved by manufacturers. The estimates came in well above $100K. 3. Which instruments should we have help moving? 4. I am confident on moving the XCT, XRD, confocal, the EDS/EBSD/STEM detectors, and even the SEMs. I am quite concerned about moving the TEMs, especially if we need any custom tools to disassemble the instruments. Has anyone moved TEM themselves? Can you please share your experience? ***Rooban Venkatesh K G Thirumalai rthirumalai@i2at.msstate.edu***

My knee-jerk reaction is to tell you that after they have spent heaven knows how much to build a new facility, they should not quibble over the costs of properly moving the equipment so that you do not end up with a collection of very costly boat anchors! I would move the smaller optical microscopes myself, in fact, I have done so, but anything that requires forklifts to move is beyond my pay scale and expertise. You may hear from third-party providers who can do this for you at a lower cost, and I'd say that may be a good option for you. Good luck and congratulations on the new facility. Lee Cohen-Gould lcgould@med.cornell.edu

Hi Lee! You are elected for the best reply (you made me laugh with your "costly boat anchors"…never thought about it, not a bad idea indeed!). Me tending to be cynical with incompetent people, I would ask the management: "Should you need someone to drive your Ferrari to the next state, would you hire the first kid you find in the street?". This way they would understand, I guess. Stephane Nizet nizets2@yahoo.com

My knee-jerk reaction is to ask why the project manager didn't include moving costs in the initial budget. Having professionals move all scopes and QC them in the new location should be part of the renovation costs. Anything under service contract must be moved or approved by the company that issued the contract. Michael Cammer michael.cammer@nyulangone.org

We just recently moved to a new lab. 1. Local crews (physical plant for the institution) usually do not do well with unwieldy, heavy objects. There are many stories from various colleagues about watching their TEM fall. 2. You will want to schedule service with the TEM/SEM manufacturer or service engineer of choice to tear down and then re-install the instruments. They will also make sure they are up and running to specifications. 3. You can contract your own riggers and shippers. They will still be expensive because of liability insurance. However, they will have the proper equipment and expertise. We asked our EM manufacturers who they trust to contract for that work. 4. If you contract the riggers and shippers, your institution *should* have insurance that you can tap into if something goes awry. You will have to double-check with your admins. John Shields johnshields59@gmail.com

1) If you need to use riggers to move the instruments over thresholds, through loading docks, or other difficult "terrain", be sure to find riggers that specialize in scientific equipment with high centers of mass. I've had success using companies that move CT scanners and MRIs for hospitals, and you should be able to receive a referral from Zeiss, JEOL, or another microscope manufacturer. This is obviously important when moving TEMs. 2) Find a retired field service engineer from JEOL, Zeiss, or Phillips/FEI/TF to quote you on the move. They will be cheaper if they are available and willing to help. 3) All can be moved yourself if you have the time, patience, and reasonable level of care. Take lots and lots of pictures of cables, label the cables with different colored tapes. Take more pictures. I agree with Michael that anything you can't lift, or crate, should be hired out for multiple reasons. All EMs are tricky to move and even trickier to get working again after a move, especially if they are over a few years old. Vibrations can kill sources, knock internal connections loose, knock boards askew, and inadvertent impacts can be fatal for both instrument and unskilled mover. No experience with moving XRDs, but they look as heavy and bulky as electron columns, and probably have nearly as much lead shielding. Chris Winkler crwinkler@ncsu.edu

You can move these instruments by yourself, but the TEM will have a very high center of gravity. Thresholds and navigating slopes can be dangerous to the microscope and crew. If you are not confident that you can move a microscope safely to the new facility, it should be left to professionals. Consider the liability of injuring yourself or students in the process. You should discuss this with your laboratory safety and facilities to make sure you are not violating safety procedures or building code by connecting or disconnecting the instrumentation. Consider the impact on your research and the amount of time it will take to do this move and restore the systems without assistance. I suspect several months downtime to move 5 EMs for a non-professional. Are you competent enough to recalibrate the microscope and spectrometers after a move? Consider the potential

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that if you move the microscopes by yourself or use a third party that you will no longer be covered by service contracts, the risk of non-covered damage and no third-party liability, and if you do not have service contracts, consider a third-party EM engineer that is experienced in that particular platform. Greg Baty gbaty@pdx.edu

Rule of thumb: If you do not know what you are doing, then do not do it. If you screw it up, then who will they blame? You. Next, if you were not hired to be a heavy-instrument rigger, then you should not be doing that job. Finally, if you have to ask the question, then you already have the answer. Having moved tons and tons of heavy equipment, I remember most the one that slid off the truck. Lucky to be alive. Jim Quinn james.quinn@stonybrook.edu

A few years back I moved a JEOL1200 out of a basement with no elevator in the building. We built a wooden ramp over a flight of stairs, and scaffolding for securing an electric winch. The equipment was then pulled up the ramp safely and intact. Another time a huge heavy crane was used to carry a TEM over the roof of a building to the street. The building was situated on a steep slope with no way of rolling the TEM to the street on wheels. A modern building should present no problem whether equipped with a loading dock or not. When renting a moving truck please keep in mind: 1) the cargo door vertical clearance should be sufficient for accommodating a TEM placed upon the moving equipment. Typically, 6+ feet of clearance is enough. Also note some cargo doors won't open fully, that is, the actual clearance might be a few inches short of advertised specs. Measure this yourself before taking the vehicle. 2) The truck must have a lift gate with a capacity of 3,000+ pounds, even if the equipment weight is less than 2,000 pounds. This lift gate should be modern, made of aluminum, and have a lifting platform with a thin front edge. Older (steel) platforms have thick front edges making rolling of heavy loads to and from the platform difficult and very bumpy (not good for a TEM). A lift gate is good to have even if both buildings have loading docks of standard height and are equipped with lifts. A couple of inches of height difference is a big problem for pulling a load in or from the truck if it has no lift gate. 3) A commercial truck is best with a metal cage inside the cargo box for securing heavy loads. Residential moving trucks have wooden rails for light cargo. The cost of residential and commercial trucks is not that different, and truck rental places have both. 4) A tall commercial truck with a modern lift gate will probably have a 24′ or 26′ long box. Not a problem, but make sure you can drive it to and from both loading points. 5) Watch for low bridges!

At the new site look at the condition of the floors, door thresholds, height of door frames, tight turns, etc. Carpeted floors require some sort of hard covering for rolling a pallet jack on them. A door threshold even 1″ high requires a thick metal sheet/plate for rolling heavy loads over it. Always secure the TEM when lifting/lowering/rolling. I use standard cargo belts with ratchets. Vitaly Feingold vitaly@sia-cam.com

My department moved into a brand-new building three years ago. Here are my recommendations based on that experience: a) For larger TEM systems, have a professional prep them for the move and reinstall them at the destination. $15K well spent. Caution the movers regarding the weight and expense involved. Truck lifts MUST be rated for that weight. No cheating. b) For the confocal system and smaller, table-top systems: You can move them, but remember that vibrations and shifting can cause greater maintenance issues than if you pay a professional. Consider each one on a case-by-case basis. Most vendors will tell you that they are the only ones that can reliably move these systems, but that is not always true. Sometimes reinstallation of a system with a periodic maintenance visit can be combined to save money. c) If you move the smaller equipment, remove or immobilize anything that moves. You'll waste hours trying to replace a lost or broken eyepiece or that special adjustable foot that came off a table. If in doubt, tape it down with low-stick tape. (I like clear packing tape.) Removing the heads and lamp housings, and wrapping them in bubble wrap will make for a smaller, more manageable, and balanced box to move. Use lots of bubble wrap and don't let things move around inside the boxes. Pack them tightly. d) Twenty-four-inch-wide plastic shrink wrap is useful. This helps to keep out dirt and snugs up loose wires on large items like TEM columns after the cabinetry is removed. e) Take pictures of every plug before removal. Label the wires in an obvious manner. Troubleshooting a misplaced plug can cost you several hours or even days. f) I have moved some scopes a short distance. You don't realize how much vibration that causes until you try it. Carts with pneumatic tires are MUCH BETTER at this. Even a one-time investment will be worth it. Buy a flatbed cart with tires and donate it to your loading dock when you're finished. Gregg Sobocinski greggps@umich.edu

Let me chuck another rock in the pond. If you have OEM service contracts on any of your instruments, you really want the OEMs involved in the deinstallation/reinstallation, otherwise you could have a much bigger bill than you're expecting after the move. The company won't consider the instrument(s) under contract until they've checked to make sure they're up to specifications. And check with your university. When I was at the University of Wisconsin they had a contract with a local heavy-equipment mover, and you had to use them, or do the job yourself. "Yourself" meaning your very own, physical self. Here at Central Michigan University, I have moved a couple of SEMs myself, but for what you're moving, you need help. When we moved to our new building, I had to move our instruments, a S/TEM, a SEM, and 2 confocals, and I happily used Central's movers. They worked closely with the company engineers (we have service contracts) and did an excellent job. So, the university people might be just the ticket. And, using them meant no issues with insurance, which is another thing to consider. Take home: Check with your university. You may not have a choice if you can hire outside help, or if you can, who you can hire. Phil Oshel oshel1pe@cmich.edu

Protecting Objectives

Confocal Listserver

Hello Core Staff and Directors. Please share how you protect objectives from being damaged by careless users (such damage is the single clause not covered by a service contract). I am really worried about our new Leica DMi8 stand that has already had the slide insert and piezo stage bent, and some objectives scratched. I calibrated all objectives for parfocality. I still am not clear how/if two sets of parfocality settings can be memorized and loaded (for universal and multiwell plate inserts). ***Arvydas Matiuka matiukaa@upstate.edu***

I think, other than putting the fear of God into students during training, there is not much more you can do, other than to save 10k USD on the side for when you get a total loss. Even if you set a hard limit, someone might change it, and/or someone might find a way

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to move the stage with some combination of insert and objective that will damage it. There are too many variables at play. To be honest, students don't read instructions or SOP documents, no matter how clear and simple they are. Even if they sign it! I tried that once and it only lasted for 3 months before I gave up. I really think it's all about the initial training and how much emphasis you put on the issue, and then, over the first few times new students start working alone, you stand over their shoulders and reemphasize the importance and make sure they are working correctly. Scheduling software that cannot be deleted may help (once a scheduled session starts, they can no longer delete the record), meaning they know that if they do something, you will know who did it. But not even that helps if you can't do anything about it even if you KNOW who did the damage. Avi Javob avijacob@gmail.com

Thanks, Avi for sharing your experience. I would add that my core mostly serves postdocs and grad students, but as you mentioned the biggest problem is with undergrads, especially ones who just want to have confocal skills listed in their CV/resume. I would also say that the "tolerance of students breaking equipment" depends on a country's cultural environment. In the USA, it is quite high, while in China it is very low. There, students are not allowed to operate expensive equipment and can only watch a technician, which is another solution for equipment protection. Arvydas Matiuka matiukaa@upstate.edu

On our Nikon Ti-E I created a macro that lowers the lenses fully and centers the XY-stage. This runs on software shutdown, and I think also on startup, in case someone uses the microscope without software. Scientists are also instructed to run the macro before changing inserts. Thus, we have prevented lens crashing for quite a few years. Stephen H. Cody stephenhcody@gmail.com

With changing times, a large segment of students and scientists have evolved into using visual cues. I found an image on the microscopy memes twitter handle and stuck it on one of our microscopes (link below) to avoid issues of users adding oil by mistake to the 20x air objective (https://ibb.co/1MHqcB0). Something that was a weekly incident, and what words in an email couldn't achieve, this meme has accomplished. Two months and incident-free. Your post made me think that perhaps, along with pretty pictures, some of the pics of microscope damage should also be posted in the core facility. Gaurav Joshi gauravnjoshi@gmail.com

In the labs I've worked in, we've lost a few lenses due to crashes, and only in one case was the person who did the damage identified. Most of the lenses were oil immersion high NA lenses on inverted systems that were smashed into the stage, but the first generation of Olympus infinity corrected lenses were also not well protected against over-oiling and other spills. We also lost a water-dipping lens on an upright due to a careless user. Three years ago, we got a Nikon spinning disk system and within a few weeks the metal on the lenses was gouged by the stage. Fortunately, none of the glass was damaged. Training did include a brief discussion of lens safety, but clearly not enough. I put macros in the software to drop the lenses and clear the XY positions on software startup and shutdown and all users had to be retrained. Since then, no additional scratches. Here are some attitudes and practices I have found. There is an expectation that we train as many people as possible as fast as possible. There is so much to learn in a training session that people cannot absorb it all. There is a culture that safety measures impede research. Biologists often have an attitude that machines are beyond them. Some may be talented biologists but have little aptitude for working with machines. I was taught physics in high school by a teacher who wanted us to understand phenomena experientially and viscerally. (And I had elementary school teachers who did all sorts of cool things with us, like build a walk-in camera obscura out of a refrigerator box and try all different types of lenses and pinhole sizes.) I get the feeling most people who walk into the microscope lab don't understand basic physics. Not enough effort and attention are put into repeated use to become truly familiar with the instrument. Microscopes are just simple tools to get pictures. They shouldn't be so difficult. You're making me consider that, before we allow anyone to use the new Stellaris arriving in a few weeks, we need to make everyone read a microscope safety sheet and test them on it. Perhaps something like a picture of a lens and explain the two most important points. 1.) No crashes. 2.) No spills. Spills are important because our Zeiss 710 NLO was nearly destroyed by spills despite the plastic spill catcher covering the nosepiece, silicon caps and scrunchies around the lenses. Michael Cammer michael.cammer@med.nyu.edu

Everything the others already said (especially the bit about saving money), plus some additional thoughts of my own.

- When moving the stage around, not only the objective in the beam path is in danger, but also the two next to it. We try to make our users aware of that problem.
- On a DMi8 a particular time of danger is when the stage is initialized and moves around automatically. We have configured our systems such that this happens not when the microscope is switched on, but only after starting the software (LASX; in the hardware configurator under stage). A pop-up screen will ask the user "do you want to initialize the stage now" or something like that, so the user must make an active choice and can check that nothing is in the way.
- Usually, the objective turret is automatically moved to the lowermost position during stage initialization (inverted DMi8), which is obviously an excellent idea. However, this does not work if the turret is not snapped into one of the objective positions. Imagine somebody wiping the oil off the metal parts and pushing the objective slightly out of position. Then, for the next user, initialization starts without the turret going down first. We learned because a user was coming to us when the stage insert was pushed up during initialization. We now ask users to switch to 5x or 10x with the touchpad before starting the software. Switching only works if the turret is snapped in.
- To avoid training of undergrad users that are not really interested in using the instrument after an introduction, we put a €200 fee on the training (converted into a prepaid account that must be used within 3 months). That seems to have helped a lot. In cases where it doesn't, at least we have the money. This helps to keep the motivation up in my team.
- During 1:1 training, we very much make the point that there are two (and only two) things where a user is in real danger of accidentally causing expensive repairs: hitting the objectives with the stage and overexposing the HyDs (detectors on the SP8 system). My impression is that most people realize that these two issues are important. I am trying to raise awareness and responsibility, but not to scare them. (Note: there are more points when liquid medium is involved and for other cases. Also, too much oil can be a problem. But giving a (small) number of "most important issues" seems to help).
- We teach our users not to put oil on the objective, but on the slide (yes, also on the inverted, before putting it on the stage), and to use a small amount. When having many slides, usually only every second

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one needs new oil, because enough remains on the lens. We also tell them to not wipe the front lens, never ever, under no circumstances, but to just put a Kimwipe (gray box) over it and suck off the oil without moving. Repeat, until it comes off dry. Wiping metal parts is okay and sometimes required. If that is not sufficient to get a good image, something is wrong, and they should call for help.

- We put hairbands around inverted oil objectives. Not sure how much that really helps, but it visually makes a point.
- I urge users to select the objective in the software, not on the touchpad: some systems have, for example, 63x oil and glycerol, on the touchpad that cannot be distinguished.
- If DAPI is used, they are told to use this to focus. If not, any bright signal (except far-red) or the green channel, even if empty, should be used: there is usually enough background and that is where our eyes are most sensitive.
- I don't think it is safer to use the 10x before going to the 63x on an inverted system where the oil must be in the beam path. I tell users to move the 63x up until it touches the oil on the slide with the coarse drive and only then look through the eyepiece and use fine focus to continue.
- The AFC (automatic focus control) can cause a problem if on "hold". We recently had a type of ibidi slide under the microscope, essentially made from optical plastic with a channel inside, and the channel on both ends will end in a large cylinder to which tubing can be connected on the top side. So, the roof of the channel vanishes when the cylinder is reached. Apparently, in this case, the reflection that the AFC was using came from the roof of the channel, and when the slide was moved such that the cylinder was in the optical path, the objective suddenly jumped up several millimeters. Ouch.
- "Not covered by service contract", if I recall, is "user inflicted damage". Scratched objectives will be the most common incident. But overloading detectors repeatedly (can be seen in logs), spilling water in electronics, or medium into the stand, etc., also fit that description. So far, we have only had scratched objectives.
- We regularly do PSF checks on the high mag lenses. Usually, we pick up problems before users complain about bad images.

Wrapping it up, I don't think you can totally avoid objective damage by users any other way than to not let them use the microscope. "The problem with making something completely foolproof is the ingenuity of complete fools" (Douglas Adams). Best you can do is to minimize the frequency. And there I am back at the beginning: Save some money for it. Steffen Dietzel lists@sdietzel.de

Oil Objective Lifetime

Confocal Listserver

Hi all, I've been told (and have read on a couple of core facility websites) that oil slowly dissolves the glue in objectives. Does anyone have a rough idea of the total amount of time an oil immersion objective can have oil on it before it seeps in? Is having oil on the objective for 24 hours straight equivalent to 24 hours spread over three days? To help make this tractable, let's assume an ideal user who cleans the objective correctly and frequently. Cheers, ***William Giang wgiang@pennstatehealth.psu.edu***

I have been doing microscopy for 30 years and have not personally encountered a problem with this. Years ago (late 1990s/early 2000s) we did have this issue with one specific lens type in an inverted configuration where user application of too much oil resulted in oil running down the side of the cap and getting to the lens barrel and down to the lens elements, but this was unusual and very specific to the lens combined with misuse. We worry more about overcleaning being a problem. As discussed, a few days ago on this listserver, lenses damaged by physical impact is not uncommon. Michael Cammer michael.cammer@med.nyu.edu

I have only heard that immersion oil can be corrosive to the cement/glue used for dry lenses, not for oil immersion objectives. Do you have a reference for the claim that the oil is a problem for immersion lenses? Elke Küster-Schöck elke.kuster.hsj@ssss.gouv.qc.ca

Here are two different sources: 1. Microcourses video (https://youtu.be/c58P4Zt9xX0?t=384): "As objectives age, it does become more likely that oil will seep through the cement and accumulate under the lens. However, it can be accelerated by using solvents too often to clean the lens". 2. Duke light microscopy core website (https://microscopy.duke.edu/guides/clean-objective): "It is important to remove the oil after use (oil traps dirt and slowly dissolves the glue in objectives)". Thanks Michael and Elke for your responses. William Giang wgiang@pennstatehealth.psu.edu

What they said. I only have 24 years, but it's the cleaning reagents, not the immersion solutions. Some immersion media will cause cancer, but that's for another thread. Oil can drip down the inner barrel, and that can wreak havoc over time, but that has nothing to do with the seals. Gary Laevsky glaevsky.lists@gmail.com

Since it's our website at Duke that was referenced, I thought I should clarify and offer one experience. I agree that it is the solvents that are used to clean oil objectives that can partially dissolve the cement holding in the front lens and may cause oil to seep in under the front lens. I will update our website to reflect this information more clearly. What is described in the Microcourse youtube description is exactly what I have seen in some circumstances (and what I've been taught/told by other microscopy experts). (Thanks for alerting me to this). We had one 40x oil objective get oil under the front lens and we returned it to the manufacturer for repair. I cannot say for sure how old the objective was because the confocal system predated my time at Duke, but I think the system was approximately 6-7 years old in 2018 when we sent the objective back for repair. In all my years of experience, I have sent back 2 objectives to the manufacturer for repair and received the objective back in good working order. We do not want our users to clean objectives and ask them to contact our LMCF staff members to help them. For instance, we do not want users taking the objectives off the turret. We ask users to carefully remove excess oil when they have completed their microscope session which included using an oil immersion lens. Of note, when I was reading the information on a couple of manufacturer's websites, it was noted that some dry objectives are not sealed to prevent seeping of oil into/under the lens, so it may not be that dry objective cement degrades more, just that the lens is not fully sealed to prevent oil seepage. Thanks for bringing this information to light. Lisa Cameron lisa.cameron@duke.edu

I've been watching this thread and thought this comment would be beneficial. When I was a much younger guy, employed by a then-Zeiss franchise in Maryland covering the central east coast, I often came across objectives that were only partially wiped off or partially cleaned. This resulted in a thin layer of oil remaining on the objective that would easily oxidize, especially overnight! This thin oxidized layer had an irregular refractive index. Then, when additional oil was added for imaging, you can imagine what happened to the image. I found the best way to clean them was to, after removing the bulk of the visible oil,

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use a strong solvent such as lighter fluid to dissolve the thicker oxidized film followed by a water-based cleaner. The petroleum cut through the oil and oxidized film (stuck to the glass) and the water-based cleaner dissolved any remaining salts and sugars that may have accidentally deposited from media or handling. It is important to note that when using a petroleum solvent, you don't saturate the wiping tissue, only make it "damp" and limit the amount of time exposure to the lens. Many times, the user would be surprised at how improved the image was. For wiping paper, I strongly suggest Berkshire, Lensx® R90 (https://berkshire.com/shop/cleanroom-wipes/nonwoven/lensx-90/ln90040624p/). It is the softest, most absorbent optical wipe I ever used. I have no interest in the company, but I haven't found anything better for lenses. Dan Fochts dan@bioptics.com

Hi Dan, just to clarify: are you talking about Type F immersion oil from Zeiss, the stuff that is still currently in use? Type F immersion oil can oxidize? We use that exclusively and never noticed anything weird when leaving a thin film on the objectives. So far, I am more worried about users scratching the front lens during "cleaning" rather than small amounts of oil remaining. Now you gave me something to worry about. Steffen Dietzel lists@sdietzel.de

Not to be too pedantic but, for cleaning an air lens, the video (https://youtu.be/Tz4Dy5D6kdw?t=203) shows a straight swipe across to top surface straight across the glass. The same surface of the cotton swab is used repeatedly. This means that any crud on the top surface is dragged across the glass and any dirt collected on the first swipe is rubbed over the lens again on the second swipe and so on. For the air lenses, we clean all the oil or other crud from the top surface around the recessed glass lens. Rotating the swab as wiping around guarantees a clean fresh surface at each location. Then we clean the recessed glass with a cotton swab. We rotate the swab in the recess as we wipe to guarantee a clean fresh surface of cotton. After one rotation wipe, the swab is thrown away. Michael Cammer michael.cammer@med.nyu.edu

I'm a bit alarmed to hear what some of you are dealing with in terms of oil films and solvents! I've just used isopropanol and a premium optical-grade tissue wipes on all our lenses for the last 16 years and have encountered none of these problems. I've been exclusively using Cargille oil, which I realize may not meet the higher optical standards many of you are aiming for, so it could be a difference in oil formulation. Just wanted to express my surprise at how vexing this problem is for some. Craig Brideau craig.brideau@gmail.com

Contrast Transfer Function (CTF) in Tilted Images

3DEM Listserver

What is the best way to evaluate the CTF of an image that has a significant amount of tilt? ***David Gene Morgan dagmorga@indiana.edu***

I think any patch-based CTF program, like the one in Cryosparc or gCTF's local mode (with evenly spaced coordinates instead of real particle locations), would work well. With CryoSPARC there's an easy method to plot the tilt axis; you can also choose a specific number of X and Y divisions for the patches. Daniel Asarnow asarnow@msg.ucsf.edu

The problem is that the tilt causes the higher resolution Thon rings to become out-of-phase, so the standard programs can't give a decent estimate of the goodness-of-fit. I vaguely remember that someone had a program that took the tilt into account several years ago, but a quick hunt for it didn't turn up anything. David Gene Morgan dagmorga@indiana.edu

The tilt can be considered as described in the CTF correction section of the paper on the 3D reconstruction of the V1-ATPase: *J Struct Biol* 135 (2001), 26-37 https://doi.org/10.1006/jsbi.2001.4395. Maybe this is what you referred to. Essentially the tilt image is divided into equal patches, the CTF determined for each patch, and a plane fit to the CTF values. The equation of this plane can then be used to determine the CTF values of each boxed-out particle based on its location in the large image and the CTF values corrected individually. Michael Radermacher michael.radermacher@uvm.edu

Both emClarity and cisTEM (recent alpha version) implement an algorithm that also tiles an image of a tilted specimen but stretches the off-axis power spectra in such a way that the Thon rings add more-or-less coherently. In emClarity, this is based on the tilt-series alignment. In cisTEM, the tilt-axis-angle and tilt-angle are fit as a part of the tilted CTF refinement. The basic algorithm is described in my thesis (online at Pitt med) and I believe in the emClarity *Nature Methods* paper. Here's a movie: https://twitter.com/cryo2go/status/1282036490378280961?s=20&t=kg1qVYhpGcqQa5z3qyAFAg. You can find some developmental versions of cisTEM and docs here: https://bhimes.github.io/cisTEM_docs/docs/sim/tutorials/tutorials.html. Benjamin Himes himes.benjamin@gmail.com

There are two separate problems here. The easier one is to simply determine the defocus in various positions of the specimen. If you only want to work with small cut-outs (such as single particles) then you use the normal CTF-correction with the local defocus. Others pointed to software that does that. If you want to work with the entire image (for example for 2D crystals) the problem is more difficult. Essentially you don't have a CTF in that case (it's not a transfer function). Look at the paper by Ansgar Philippsen: *Ultramicroscopy* 107 (2007) https://doi.org/10.1016/j.ultramic.2006.07.010 if that's what you're after. All the best, Philip Koeck koeck@kth.se

Just to clarify, the patch methods don't require particle locations. The gCTF method requires particle locations as input but there's no need for them to be real particles; it too just estimates the CTF on the patches. David, what do you mean by the goodness-of-fit estimates being incorrect? In these methods a goodness-of-fit measure is used to pick the optimal defocus values in the patch, and these values do give superior reconstruction resolution and appear to accurately define the real tilt axis. Daniel Asarnow asarnow@msg.ucsf.edu

Maybe goCTF is the program you were thinking of? https://doi.org/10.1016/j.jsb.2018.11.012 and https://www.lsi.umich.edu/science/centers-technologies/cryo-electron-microscopy/research/goctf. Guillaume Gaullier guillaume.gaullier@icm.uu.se

If you do subtomogram averaging in Relion-4, estimating these high-tilt CTFs over the entire micrograph is probably not that important if you optimize defoci in a CTF refinement job after averaging. There, the entire tilt and sample geometry are taken into account. Sjors Scheres scheres@mrc-lmb.cam.ac.uk

Perhaps CTFTILT? https://grigoriefflab.umassmed.edu/ctf_estimation_ctffind_ctftilt Mike Strauss mike.strauss@mcgill.ca

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As a practical matter, this issue is resolved by the patch approach. How cryoSPARC averages the patch fit quality has not been described, but tilted images can be filtered very well in my experience. The local defocus patch estimates are also generally very accurate - it's common for CTFFIND to fit a highly tilted image to 6-8˚, but patch fits of the same image extend to 3-4˚ (the programs use the same definition of the fit resolution), and single-particle estimates improve on the patch values for large particles like ribosomes or the 20S proteasome. These notes are all from a single-particle perspective. Daniel Asarnow asarnow@msg.ucsf.edu

This paper from 2007 in *Ultramicroscopy*, "The contrast-imaging function for tilted specimens" (https://doi.org/10.1016/j.ultramic.2006.07.010) seems relevant to your question! Michael Elbaum michael.elbaum@weizmann.ac.il

That's the paper by Ansgar Philippsen et al. I mentioned. The theory presented there is only valid for thin specimens, but it is not limited to small patches, so it describes contrast "transfer" (not a transfer function!) for the whole image. Philip Koeck koeck@kth.se

Thanks to all for their input. Prodded by several of you, I realized that I was trying to remember a program called ctftilt that was released in parallel with ctffind3 many years ago. From the responses I received, it appears that goCTF and the version of ctffind in the development branch of cisTEM are more recent versions that do what ctftilt did. It is also clear that some packages have built-in ways to process single particle data acquired from a tilted grid. I was looking for something to evaluate the images themselves, before doing any further processing. I did not try any of these packages, and it is entirely possible that they generate diagnostic data similar to the results from ctftilt (and the others listed above). Again, thanks for all your input, and I hope this summary isn't a waste of your time. Daniel Asarnow asarnow@msg.ucsf.edu

New Spectra Viewer

Confocal Listserver

Dear all, I would like to draw your attention to our new fluorophore database and spectra viewer. You might be wondering why we need yet another spectra viewer, but I think we included some very useful features:

** The viewer uses a large database with more than 550 fluorophores and spectra of more than 250 optical elements (filters and mirrors).*

** The database contains two- and three-photon excitation spectra for more than 150 and 10 fluorophores, respectively. We are regularly adding additional spectra. Please let us know if you have spectra that are currently not included.*

** Custom spectra can be uploaded and displayed along with the spectra contained in the database.*

** Plots can be displayed using normalized or absolute values on a linear or logarithmic axis.*

** In addition to the "standard" spectral plots the app provides a "2D viewer" in which excitation and emission spectra are combined into 2D contour plots. This is particularly useful when displaying excitation/emission spectra for various fluorophores simultaneously.*

** Several additional fluorophore properties are contained in the database and can be accessed through a fluorophore browser (a table with various search and filter functions) or visualized using a 2D scatter plot. Up to four properties can be visualized simultaneously using the marker's x- and y-coordinates as well as their size and color.*

** The viewer works quite well on mobile devices. You can find the viewer here: https://public.brain.mpg.de/shiny/apps/SpectraViewer/ and additional information here: https://brain.mpg.de/326043/spectra-viewer. I would be happy to get your feedback (questions, comments, bug reports, feature requests …).* ***Stephan Junek stephan.junek@brain.mpg.de***

Thank you for sharing this. I think the additional functionality of the 2D viewer, and the scatter plot could be helpful, especially for planning some multilabel spectral imaging experiments. Silas Leavesley leavesley@southalabama.edu

Thank you for sharing. It looks good. One suggestion: could you link with the fluorescent protein (FP) database (https://www.fpbase.org)? That resource has information about each FP, including a unique identifier. As part of QUAREP.org we are working to revise the Microscopy Metadata specifications developed initially by 4DN-BINA and OME which were recently published on *Nature Methods* (https://doi.org/10.1038/s41592-021-01327-9). Caterina Strambio caterina.strambio@umassmed.edu

This is awesome and very helpful. I am wondering if we can also start including other molecular (for example, opsin's) spectra in such databases. That will be a great resource for optogenetic experiment/setup designing. Anuj Sharma anujjnu@gmail.com

Along the same vein of discussion, I thought this would be a good place to post links to the many different spectra viewer tools online that I've found:

1. Thermo Fisher Scientific SpectraViewer: https://www.thermofisher.com/order/fluorescence-spectraviewer#!/
2. Omega Filter's Curvomatic: https://www.omegafilters.com/curvomatic
3. Max Planck Institute Spectra Viewer: https://public.brain.mpg.de/shiny/apps/SpectraViewer/;
4. Chroma's Spectra Viewer: https://www.chroma.com/spectra-viewer
5. FP Base (fluorescent proteins only): https://www.fpbase.org/spectra/
6. Semrock's Searchlight: https://searchlight.semrock.com/
7. Fluorophores.org (click on dye to see spectra): http://www.fluorophores.tugraz.at/substance/
8. BioLegend Spectra Analyzer: https://www.biolegend.com/spectra-analyzer

Jason Kilgore jason.kilgore@thermofisher.com

I wanted to quickly point out that: "FP Base (fluorescent proteins only): https://www.fpbase.org/spectra/" is not fluorescent proteins only (there are huge numbers of dyes) and it has some neat microscope setup options to boot. Mike Nelson msnelson@gmail.com

Trouble Seeding Cells on Gold Grids

3DEM Listserver

Dear all, in our lab we have grown mammalian cells successfully on holey carbon R2/2 200 mesh gold grids (Quantifoil) for some time now. However, a few months ago we started having problems with cells not attaching, spreading, or looking happy on the grid. Mitochondria stained with MitoTracker sometimes looked normal but were often smeared or bloated. This differed greatly per experiment. We hypothesized that something might be wrong with

the grids. To determine what was going on, we conducted an experiment where, in parallel, we seeded cells on glass, continuous carbon (gold, 200 mesh, Quantifoil), or the presumably faulty/toxic R2/2 (gold, 200 mesh, Quantifoil) and stained with MitoTracker. Some grids were washed either in ethyl acetate/ethanol/MilliQ or ethyl acetate/acetone/chloroform and ethanol/MilliQ prior to seeding. The mitochondria of the cells seeded on glass and the continuous carbon looked fine, but the mitochondria of the cells on the R2/2 grids both unwashed and for the washing protocols looked smeared or bloated. We wonder if anyone else has had trouble with cell seeding on grids or has suggestions on other washing protocols? Alternatively, would it be useful to try Protochips c-flat grids instead? Kind regards, ***Leanne Jager l.a.h.dejager@uu.nl***

I normally wash grids with acetone and ethanol and incubate them in cell culture media overnight in a cell culture incubator. The next day, I replace the media and seed the cells. For some cell types, I coat the grids with poly-lysine or gelatin. How long have your cells been in culture? I had a similar experience where cells would not spread well on holey carbon grids while looking fine on glass or plastic when they reached a certain passage number. Daniel Serwas daniel.serwas@berkeley.edu

We had a similar problem when changing from one batch of grids to another where our cells (neurons) did not grow well on Quantifoil grids. Turned out the reason was the manufacturer changed a washing step, but it took almost a year to get good grids again. Vladan Lucic vladan@biochem.mpg.de

You could try flame sterilization of grids. I used this for primary neurons and no coating was needed. Just sterilize shortly before seeding cells. Julika Radecke julika.radecke@diamond.ac.uk

Question about Human Epidermoid Cancer Cell (HEp-2) Morphology

Microscopy Listserver

We have a TEM sample of control HEp-2 cells. In thin sections, the cells have areas of various sizes where it appears the cytoplasm has been "removed," for lack of a better word. At low magnification our first thought was that the cells were showing vacuolization. However, these areas are not membrane bound. Within the areas, the faint material looks like the surrounding cytoplasm. These cells have several lysosomes in various stages, but otherwise the cells appear healthy. I do not think it is stain, fixation, or other preparation artifacts. Some cells have many of these areas while others have very few. There is no evidence of bacterial or viral contamination. Nothing was done experimentally to these cells, as baseline morphology is needed before doing experiments. Is it possible to have digestion or degradation of the cytoplasm in the absence of any kind of membrane bound digestive vesicle or vacuole? All help is appreciated. ***Tom Bargar tbargar@unmc.edu***

How many times were these cells passaged? Many moons ago I worked with HEp-2 cells, and I remember that they get really ugly after repeated passages. I was using them in light microscopy, and they had very large accumulations of "stuff" in them. The solution was to use a lower passage number. I know that doesn't answer your question directly, but it may not be anything you did once they were in your hands, rather that the cells were "too old". Lee Cohen-Gould lcgould@med.cornell.edu

Hep-2G cells are frequently filled with non-membrane-bound lipid droplets. Many examples are out there via Google images. Thomas Phillips phillipst@missouri.edu

What buffer was used? Phosphate buffers may cause uneven extraction of cytoplasm. Glutaraldehyde generally is less extractive. Phil Oshel oshel1pe@cmich.edu

The only thing I can think of is that the cells showing this morphological feature are dying. In this case, one can see strange intracellular structures which relate to nothing you expect to see. If this happens in only a few cells, it is possible to miss them in the light microscope. However, if most cells look like that (most cells are dying), this should also be recognizable under a light microscope to any person acquainted with these cells. I would ask these questions: How old is the cell culture? How many passages? Are the cells mycoplasm free? Another reason might be extraction during dehydration. In this case I would consider contamination in the dehydration solutions. This is easy to verify: prepare other, well-known cells in your laboratory with the same method and the same products and if they show the same "artifacts", you have found the suspect! Stephane Nizets nizets2@yahoo.com

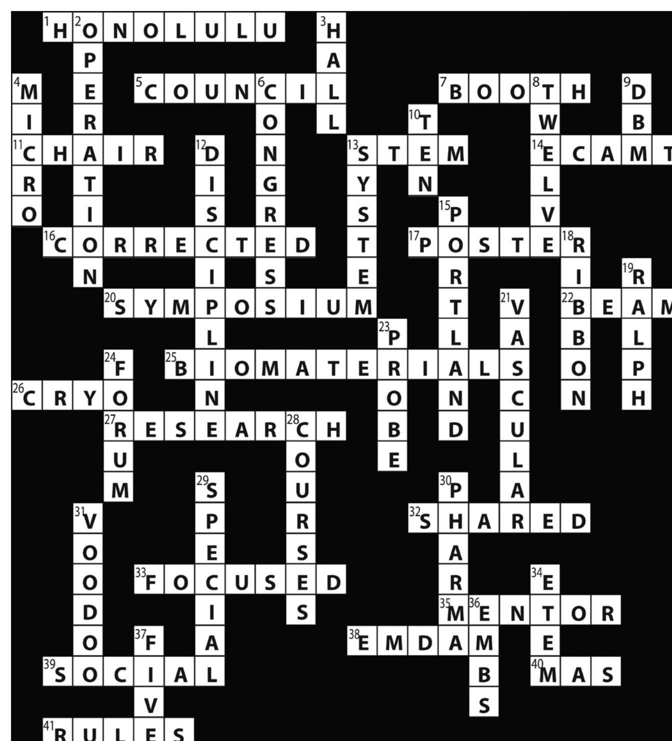
Do these cells have glycogen granules? Did you process them with uranyl acetate *en bloc*? Uranyl acetate partially removes glycogen and creates empty areas in the cytoplasm. Hilda Amalia Pasolli amaliapasolli@gmail.com

Someone previously mentioned lipids. If OsO_4 was not used, then lipids would be extracted in subsequent solvents and liquid plastic. Jan Factor jan.factor@purchase.edu

MT

Crossword Puzzle Answers

See puzzle on page 58.



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