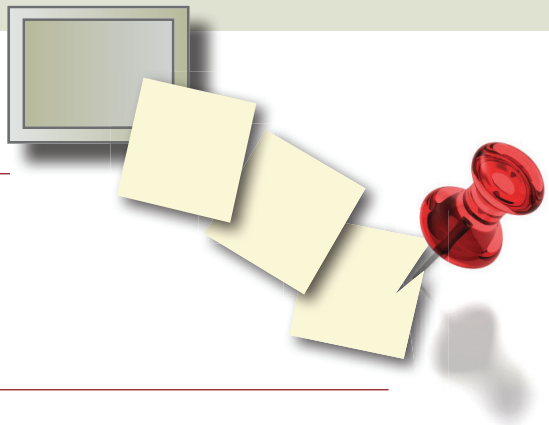
NetNotes



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Selected postings are from discussion threads included in the Microscopy (http://www.microscopy.com) and Confocal Microscopy (https://lists.umn.edu/cgi-bin/wa?A0=confocalmicroscopy) Listservers from July 1, 2019 to August 31, 2019. Postings may have been edited to conserve space or for clarity. Complete listings and subscription information can be found at the above websites.

Techniques

Confocal Microscopy Listserver: Killing cells in a zebra fish

We have a user who wants to kill specific GFP labelled single cells in a zebra fish and follow the developmental consequences. Multiphoton or confocal used to zap a selected area was the suggestion but is bleaching the GFP sufficient to damage the cell. How can we determine if the cell is dead/damaged: use of trypan blue in fish? We also need to assess the extent to which surrounding cells are also damaged. Are there any photoactivatable toxins? Ideas, references, suggestions would be greatly appreciated. ***Jeremy Adler jeremy.adler@igp.uu.se***

Photodynamic therapy (PDT) does exactly this. I've used verteporfin in the past (http://dx.doi.org/10.1088/1612-2011/11/11/115605), but there are plenty of alternative PDT agents. Regarding the assessment of the cell, is it possible to sacrifice the fish and perform a LIVE/DEAD stain? Or if you need it still alive, bleach the GFP and see whether the GFP intensity recovers over time (suggesting that protein synthesis is still occurring). Chris Rowlands c.rowlands@imperial.ac.uk

You might consider using "Killer-GFPs" like Killer-Red or Supernova. They are used for CALI, chromophore assisted laser inactivation. If the cytoplasm is full of it, then the irradiated cell should die. You might be able to use really low laser powers. I don't know about fish, but in cultured cells Calcein is a good live/dead stain. Elisa bioimaging@uni-konstanz.de

Yes, we have photosensitized cells with GFP. We used a 405 laser line to ablate pronephric kidney tubules. You can use propidium iodide to track the cell death. Here's one of our papers on this: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4092191/ Iain Drummond idrummond@mgh.harvard.edu

I recommend using a mitochondrial or nuclear-targeted Killer-Red for that purpose. You will need a pretty modest 555 nm light flux to zap targeted cells into apoptosis. There are probably newer and better genetically targeted photosensitizing agents, but that's the one that I've used. Timothy Feinstein tnf8@pitt.edu

I have not done this, but in this paper I read a while back (Liu et al., Immunity 2016 <https://www.sciencedirect.com/science/article/pii/S1074761316300978>), they used 2-photon microscopy (800 nm) to kill single endothelial cells in zebra fish brain to study blood vessel repair using intravital microscopy. From their methods: "Laser Microsurgical Cell Ablation, Photoconversion, In Vivo Time- Lapse, and FRET Imaging For Laser Microsurgical Cell Ablation", the target endothelial cell or macrophage was focused in a single confocal plane and irradiated for 3 s by a multi-photon laser at 800 nm (Chameleon Vision2 Laser System, Coherent). Hope this helps. Henrik Yiradati henrik yiradati@gmail.com

You can try using a laser-capture microdissection microscope. We have used it to cut a zebrafish axon and the fish survived the procedure. Esther G.L. Koh lsiailip@gmail.com

It depends a great deal on how deep the GFP+ cells are into the animal and whether the goal is to kill individual or all GFP+ cells. We had great success using blue light output from a dye laser (Andor Micropoint) in killing hair cells in 2–3 day old larvae: https://www.cell.com/developmental-cell/fulltext/S1534-5807(17)30864-X. But these are cells are quite superficial. For broader killing, the ROS generating fluorescent proteins that others have mentioned should work well as long as the light exposure required doesn't also cause off-target toxicity. For killing individual cells deep inside of tissue, your only real bet is a femtosecond laser. For a recent implementation of this, see this paper from the Ahrens and Keller labs: https://www.nature.com/articles/s41592-018-0221-x

Our experience both with femtosecond and nanosecond lasers for killing single cells has been to use damage accumulation—a long train of low energy pulses to bleach a visible spot in the nucleus, but not so much that the entire nucleus or cell bleaches. In our hands this has been quite important for limiting off-target damage. It's important for there to be residual signal so one can actually assess cell death- typically one would expect to see the nucleus either condense or fragment anywhere from 5 minutes to 45 minutes after ablation. Hair cells seem to be cleared within minutes after death so, depending on your time-lapse imaging rate, the corpse can simply disappear between time points. Acute insults like laser-induced cell killing can also cause significant off-target damage on-axis with the laser, so it's also important to watch cells above and below the target for signs of death or damage as this can potentially confound your experiment. Please feel free to reach out if I can provide any more detailed advice! Pavak Shah pavak.k.shah@gmail.com

Microscopy Listserver: TEM study of beam sensitive amorphous samples

We have a FEI Talos F200X TEM and I am having trouble measuring the composition and doing spectral imaging (EDS map) of an amorphous film (Si, Mg, Fe-oxide) using EDS. I have tried a large gun lens value (STEM mode: extraction voltage 4500, gun lens 6) and large spot size (6–8,) but the sample is destroyed after few minutes of mapping (20–50 microsecond dwell time). Does anyone have experience

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in studying beam sensitive samples and the parameters (extraction voltage, gun lens, spot size) that I can use to decrease damage in the sample before I can get reasonable counts in the EDS map and spectrum. Does using a large spot size of 3–4 nm in the STEM mode help? I would greatly appreciate your views and suggestions. ***Surya Snata Rout surya.rout@tuhh.de***

The parameters you are trying to change (gun extraction, spot size, etc.) will only have a marginal impact on beam damage - you have to remember that in order to get an X-ray emission you have to ionize your specimen, and this by definition damage!. So to get the counts you want damage may be unavoidable. I would say that spot 8 and gun 6 are not normally considered 'large' values for your instrument, and as you increase the extraction voltage you get more electrons (with all other things remaining the same), so you may not be reducing your current as much as you think. Try reducing the extraction (say down at 3800) and increasing to gun 8. Are the features you want to map on the same scale as your probe size? If you are mapping large (nm scale) features with a sub nm probe you may get some joy spreading the beam (as you suggest), but I have not seen this work very often. However, you may get more mileage and minimize the impact of beam damage by trying a lower voltage—run at 80, 100, or 120 kV. This may work if you are limited due to knock on damage. Note, however, that in some cases this will make damage WORSE, when the damage mode is dominated by electron beam heating (cross section increases with lower voltage). If this is the case then alternative strategies could be to cool the specimen with LN2 or especially if your material is a non-conductor, coat the exit surface (bottom) with a very thin (1 nm if possible) carbon coating. This will provide a conduction path for heat/charge. Since I presume you are already at 200 kV on your Talos 200X you don't have the option of increasing the voltage, but 300 kV can be much better then 200 kV in these cases. The main message is that beam damage is completely specimen dependent, as such the usual approach is simply to try a number of options. Good luck. Matthew Weyland matthew.weyland@monash.edu

Microscopy Listserver: Freeze substitution of ice cream

I received some ice cream samples for freeze substitution and lowicryl HM20 embedding. My problem is the samples are floating on the surface of the substitution cocktail and do not sink into the bottom of the vial. Does anybody have experience with this kind of experiment? How can I keep samples submerged in the solution and embedding medium to get good substitution and then UV polymerization? Thank you! ***Gang (Greg) Ning gxn7@psu.edu***

I am not sure if you need SEM or TEM, but I have had excellent results with cryo-SEM and ice cream. Debra Sherman dsherman@purdue.edu

I am not at all sure whether FS+embedding in a resin makes any sense with ice cream samples, at all. I would go for anything like cryo-fracture (plus some short freeze-etching / gentle freeze-drying at -80/90/100°C in high vacuum??) , and then go to a cryoSEM (cryo-shuttle), or—if available—a final coating with carbon and/or Pt/C (at or below -100°C) , i.e., like freeze-etching any other bio-sample. Exactly like Debby's suggestion Reinhard Rachel reinhard.rachel@biologie.uni-regensburg.de

Hello, Reinhard. Thanks for the input. Yes, we do have a good cryoSEM setup and have done ice cream with it in other projects. However, for this specific experiment, we want to know the TEM structure of the ice cream and immunolabeling to identify specific components in it, maybe do CLEM. Gang (Greg) Ning gxn7@psu.edu

Hi Greg, I see your point (although not clearly, yet). I am quite worried about keeping the specific structure of ice cream as it is after dehydration, and I do not see a route to keep this structure even by freeze-substitution and embedding. Yes, one drawback for FF: in fractured samples, you get ONE sample (or two samples / replicas). You cannot section/fracture once again. And for CLEM, I do not have a method. A note: Immunolabeling can be done very nicely on fracture replicas (Severs NJ and Robenek H (2008) Freeze-fracture Cytochemistry in Cell Biology. Methods in Cell Biology 88: 181–204) and (Kazushi Fujimoto 1995, Freeze-fracture replica electron microscopy … in J. Cell Science 108, 3443–3449) and I have more literature on this. In fact, the efficiency of labeling on fracture replicas is often higher than on sections. Good luck! Regards. Rachel Reinhard reinhard.rachel@biologie.uni-regensburg.de

Use a dense open-pore polymer sponge/stopper (like that used to cap Drosophila culture bottles). Put the sample in the solution and push in the stopper until it pushes the sample below the solution surface. The sponge will allow the solution to come in contact with the sample surface touching the stopper, insuring complete infiltration. This can be done for all steps of the processing, but you'll have to change the stopper along the way, otherwise there'll be carryover from one step to another. The topmost edge of the sample may suffer from incomplete polymerization using UV, but that bit could be considered sacrificial … it won't be pretty when sectioned anyway. If you don't have the foam/sponge, wadded-up lens paper should work. Philip Oshel oshel1pe@cmich.edu

Hardware

Microscopy Listserver: Cleaning the wehnelt cap

We are in the middle of troubleshooting gun instability issues on our 2010 TEM and one simple task was to clean hundreds of hours of lanthanum "tarnish" from the Wehnelt cap. Not so simple! We had reasonable success with metal polish on the easy to reach surfaces near the cap aperture, but a devil of a time cleaning the INSIDE of the aperture that contains a rather stubborn layer of tarnish. We are hesitant to manhandle the cap so I thought I would seek wisdom from the community. Any suggestions for methods or products would be greatly appreciated. ***Tom Williams tomw@uidaho.edu***

I can pass along some tricks from FEI and JEOL service engineers. There are a couple of methods to remove the stubborn LaB6 residue from the Wehnelt. One method is to soak the Wehnelt in full strength Micro-90 cleaner overnight, and follow up with a few hours of ultrasonication (using a quality sonicator!) in fresh Micro-90 followed by the metal polish compound. That may not be enough if the Wehnelt hasn't been cleaned in a few filaments, so another method is to scrub the Wehnelt with Bon Ami powder cleanser and a soft toothbrush or other gentle scrubbing tool and again with the metal polish. If you're really struggling, a Dremel tool with a cotton Q-tip on low speed coupled with metal polish is a bit more aggressive. You probably will not be able to remove everything no matter what you do, and you should not see any negative effects from a little bit of LaB6 residue around the aperture. I'm sure others have alternative recipes that will help. Good luck. Chris microwink@gmail.com

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Here is the procedure I have used for decades on thermionic e-gun parts. It works beautifully every time with lasting results.

Materials and tools:

- polishing paste such as Wenol or Pol or Pikal
- high-density cotton fabric such as cotton lintless cloth
- cotton Q-tips on wooden sticks
- fine grade sandpaper, 1000 or higher
- ultrasonic cleaner
- 3 glass beakers of suitable size
- medium size tweezers
- sharp scissors
- acetone hardware store grade
- dry compress gas (duster can)
- powderless latex gloves
- wearable eye loops or high power reading glasses

Procedure:

- disassemble Wehnelt completely to the last screw
- use polishing paste on a cloth to polish open surfaces
- use Q-tips to polish tight spots
- use wooden sticks of Q-tips to polish tightest spots such as apertures
- use sharp-pointed wood sticks (cut with scissors) for reaching inside tightest spots such as aperture
- clean excess of polishing paste with cloth / Q-tips / wood tips
- sonicate in three consecutive acetone baths, 5+ minutes each, do not let them dry between baths
- when done dry parts in air at room temperature for up to 10 min.
- inspect in bright light wearing high power glasses or eye loops
- assemble and align wearing powderless latex gloves

Comments:

- Wehnelt apertures down to 300 µm can be cleaned this way (but not obj. /C2 SA apertures).
- Only parts in "direct view" of the cathode tip and "direct view" of the emission chamber cavity must have a shiny polished metal surface. Wehnelt assembly parts facing each other or the porcelain insulator should be "generally clean," yet some minor oxidation is okay.
- Particles of hard polishing material will slide down while in the ultrasonic bath. Position parts so that concave surfaces face down and convex surfaces face up as much as possible. If not possible then turn parts of complex shape a few times. This way polishing material will not accumulate inside Wehnelt components.
- Move parts from one bath to the next with tweezers, while ultrasonic power is on, and blow excess acetone off with compressed gas before placing part into the next bath.
- Why acetone? Why hardware-store grade? Acetone dries off quickly. Acetone from a hardware store, whatever is in it, is 100% VOC and drying with zero residue—all that is required from it for this procedure. And it's cheap.
- Sandpaper is usually not needed, unless for cleaning of previously neglected parts. If you have to use sandpaper just be careful and take it easy, and always follow with polishing paste.

Good luck! Vitaly Feingold vitaly@sia-cam.com

Big thanks for all the great advice and info on ridding my Wehnelt cap of its unsightly LaB6 grunge. Success on the grunge. Still battling other gremlins, but the cap is clean! Thanks! Thomas Williams tomw@uidaho.edu

Microscopy Listserver:
Chiller replacement on T12 TEM

Our Haskris R075 water-cooled chiller for our Thermo/FEI T12 TEM needs to be replaced. Has anyone switched and/or have feedback on an air-cooled chiller? We would love to be able to bypass our university chilled water if there aren't any drawbacks that we can live with. I've gathered that the air-cooled chillers are a bit noisier. ***Melissa Chimento mchimento@uab.edu***

We have done this once. We will not do this again. We even shipped the air-cooled version to the manufacturer to get it rebuilt to the water-cooled version. Here during a hot summer, in the room where the air-cooled chiller was positioned, the temperature was above 30°C (no air-conditioning possible!!), and thus, the chiller was not able to provide the water temperature (18°C) needed/desired for the TEM (for the CM12, i.e. the earlier version of the T12). In your case, if the room temperature with the chiller is tightly controlled and ideally air-conditioned, you may consider an air-cooled version. Yes: the air-cooled version is (a) cheaper, (b) produces a lot of "warm air" (logically), and (c) is noisier than the water-cooled version. As usual: it depends. I can understand why you want to bypass the University's chilled water. Here we have water filters in front of the chiller input in order to prevent the dirty university chilled water from blocking the heat exchanger inside the chiller. Rachel Reinhard reinhard.rachel@biologie.uni-regensburg.de

Air cooled chillers present their own problems (extreme heat and extra noise). I've seen them work when the microscope room shares an outside wall (I believe the outside-location chiller will suffer more wear), and I had one installed in a penthouse directly above the TEM microscope. The heat is pretty extreme, though. We were running a chiller using city water at our previous, 100 year old building location. You'll always need a pre-filter whether using city water (mostly rust) or university water (mystery black substance). When using city water, you'll need a drain for the chiller to dump the warmed water. I'm not sure if anyone over here determined how much water was dumped during normal operations. It was a legacy system when I came on board. Gregg Sobocinski greggps@umich.edu

At ASU, we had a water-to-water heat exchanger so that the university's dirty water did not go through the chillers. A. John Mardinly john.mardinly@asu.edu

Here at UCLA we have the water chiller hooked up to the house chilled water to cool the compressor. Odd pink water they have here. It was piped to have an inflow and outflow for the university water system. It has worked for quite a while on both our older EM208S scope (20 years) and out Tecnai G2 (3 years). Desert Rat desertrat99@verizon.net

For 25 years now I have used an air cooled "Van der Heiyden Kühlmobil" water chiller in series with a heat exchanger cooled by the house chilled water. The chiller has a simple modification: The compressor is controlled by an external thermal switch. In normal operation water from the microscope is chilled by the heat exchanger. If the temperature at the outlet of the heat exchanger is low enough, the compressor of the chiller is off. The chiller only pumps the water to the microscope. If the temperature at the outlet of the heat exchanger is too high, the thermal switch starts the compressor (for 24 hours

to avoid switch on/switch off) and water is chilled by the air cooled chiller. Never place the air cooled chiller in the microscope room. Air cooled chillers should be placed in an environment with low ambient temperature (basement…) Best wishes. Winfried Send winfried.send@gmail.com

Laboratory Design

Confocal Microscopy Listserver: Design of single, open workspace

I want to request the help of this fabulous community in the construction of our new facility that will gather at least seven instruments in a single workspace (Light Sheet, Seahorse, 2 Confocal microscopes, 2 epifluorescent microscopes, and 1 Cellomics system). I would like this space to be a unique open space (something similar to an emergency room of a hospital). Each microscope will separated with good quality curtains and with well-directed light on the equipment. Moreover, when no equipment is used I would like to have a beautiful view of all the equipment at the same time. I have several questions that I hope you can help me with. We will greatly appreciate it since we are making an enormous investment and we have no references for this type of open laboratory.

- *Have any of you considered or built a space of this style with several microscopes in a single open space (only separated with curtains)?*
- *Do you know any curtain and curtain rails ideal for this type of construction?*
- *Concerning lighting, do you know of lamps or bulbs that will focus the light over the microscope only?*
- *Do you know of some type of photochromatic glass or similar material that allows showing of microscopes but that blocks the light completely when we are working and prevents light from entering from outside?*

Thanks. ***Jorge Toledo orgetoledoh@gmail.com***

I remember seeing something similar in MPI-CBG, a huge room with many machines in it, separated by curtains. But, each room also had a regular door for entry; think of it as a big room inside a building, so that the room has a number of doors from each side. One microscope was located behind each door. The individual workspaces were still divided by heavy curtains, but you don't normally have to move them at all. It's more problematic when users need to pass through the curtains, as they tend to be heavy and cumbersome. This results in the curtains being open all the time. But that's okay, too. You don't really need a dark room for Seahorse, not even for a confocal. It's more critical with epifluorescence scopes and TIRFs, and paramount in case of multiphoton imaging. With lightsheet it depends. The Z.1 can sit on your office desk, but the Phaseview alpha would definitely benefit from a dark room, preferentially vacuum and suspended in zero gravity. Curtains and rails: I haven't found the right ones yet. With rails made of short segments the curtains tend to get stuck at the segment boundaries, so the skill of the person mounting them is important. For lights, dimmable fake fluorescent ceiling lights (LEDs) are okay (assuming your curtains go all the away to the top). It's great if users can control the light while sitting by the scope. What glass? I have never seen that. I would avoid glass in dark rooms. Good luck with your new facility! Zdenek zdedenn@gmail.com

If your curtains go all the way up to the ceiling you will need to consider the heat getting trapped in each pocket and need separate air supply to each curtained area. If the curtains do not go up to the ceiling the heat should mix within the room, simplifying overall climate control needs. The drawback is limiting light into the curtained areas when they don't' go to the ceiling. You might want a hybrid approach, where you have some curtains placed lower and one or two areas up to the ceiling with extra ventilation. In this case, a system like a multiphoton, which requires darkness *and* generates a substantial heat load, will be satisfied. Craig Brideau craig.brideau@gmail.com

We had curtains in the lab I was in for grad school for a while. They were black-out curtains with an additional valence that hung from the rail to provide overlap and to minimize light. They were just okay. Sometimes they were cumbersome, but we worked around it. One thing to note: the shed. Especially with the ones that often get moved back and forth. If you have any specific instruments that suck in large amounts of air with poor dust-handling capabilities, then the optics in those systems could get dirty quickly. I guess you could maybe try for some other type of curtain material, maybe vinyl like really thick black shower curtains, if such a thing exists. Good luck and best wishes for a successful design! Silas Leavesley leavesley@southalabama.edu

Not sure how this frames up, but our biosafety folks are not keen on any fabric materials in our imaging spaces if in Level 2 labs / spaces. Materials must be hard surfaces or washable. So fabric blackouts are a no-go. Christopher Yip christopher.yip@utoronto.ca

It's the same here. They made us paint a wood frame that had a faraday cage with lacquer just so it could be scrubbed down. Silas Leavesley leavesley@southalabama.edu

No cardboard boxes allowed in our level 2 spaces either. Christopher Yip christopher.yip@utoronto.ca

Our facility is completely open concept. I love it and would never go back to individual rooms. The facility is always quite dark (I have the bruises to prove it) as each microscope is illuminated by its own remote controlled spot lights that are usually off except when someone is loading/unloading a sample. Each scope is separated by cheap, potable office/cubical dividers. The dividers are easy to move around when we exchange equipment and they block what little stray light there may be from a neighboring system's spot lights. The one exception is our multi-photon platform. It is housed in a corner of the facility behind a floor to ceiling black curtain. Additional ventilation exists within this space, although after a day of constant imaging it is often a few degrees warmer than the rest of the room (all ventilation is HEPA filtered and low flow). The open concept encourages collaboration and leads to all kinds of "Wow, what is that!?" types of discussions. I can honestly say, it's brought a number of labs together. Douglas Richardson ds.richardson@gmail.com

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