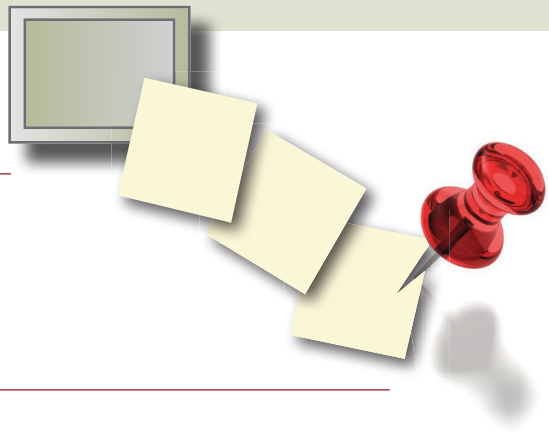
NetNotes



Bob Price
University of South Carolina School of Medicine

Bob.Price@uscmed.sc.edu

Analysis of Apoptosis

Confocal Listserver

We would like to analyze cell death over a 24h time lapse in cultured cells that already express Venus. We would appreciate any recommendations for reagent that can last at least 24h with an EM/Ex spectrum that does not overlap with the spectrum of Venus. Any other advice is also very welcome. Thanks in advance. ***Verona Villar vvillar@umh.es***

Not sure about 24h, but I think CellEvent is used for long incubations. Mike Model mmodel@kent.edu

I don't know if this will be helpful but I'm linking to a video (https://www.dropbox.com/s/okmxszk4vng3010/Dying%20cells.mp4?dl=0) showing a cell death experiment where we put calcein AM and propidium iodide (PI) in the media and also stained the cells with Hoechst 33342. As the cells die, the breakdown of the plasma membrane can be tracked by noting when the calcein stain disappears, which is followed almost immediately by breakdown of the nuclear membrane and PI positivity. This was not a 24-hour experiment, but a similar protocol could work for you. You need to ensure that the cells have a good control for phototoxicity and any other factors which can cause cell death. Chris Guerin chris.guerin@irc.vib-ugent.be

Along the same lines as Chris's suggestion, DAPI also makes a great viability marker. Benjamin Smith benjamin.smith@berkeley.edu

Depending on what stage(s) of cell death or apoptosis you are interested in tracking, there are a number of options to consider, each with their caveats beyond a quick thread here. Here's a Methods paper (*Measuring Apoptosis at the Single Cell Level*, https://doi.org/10.1016%2Fj.ymeth.2007.11.007) I worked on with a few fantastic colleagues in Doug Green's group more than a dozen years ago. Here's another option from an Apoptosis Methods book that you might have access to (no free access that I can find) from that same time period: *Live to Dead Cell Imaging* (https://doi.org/10.1007/978-1-60327-017-5_3). Sam Connell samuel.connell@gmail.com

Thank you all very much for your advice and for sharing your experience in this field. Best regards, Verona Villar vvillar@umh.es

Fluorescent Indicator for Membrane Damage

Confocal Listserver

Does anyone have suggestions for indicators of membrane damage (possibly short-lived) in live cells? We are trying propidium iodide (PI), which is not supposed to enter intact live cells but may enter a damaged transiently damaged membrane and then light up in the nucleus. The nice thing about PI is that it is not fluorescent until it associates with DNA or RNA. Are there other methods that people on the list can suggest? Thanks in advance. ***Aryeh Weiss aryeh@cc.huji.ac.il***

I am not sure if this might help. Phosphatidylserine is not exactly an indicator of plasma membrane damage, but with stress and loss of homeostasis it is externalized to the outer leaflet of the plasma membrane. This can be easily visualized with Annexin V conjugated to a fluorophore (there are many commercial sources of this reagent). This happens before propidium iodide can get into the cells and label DNA. Javier Diez Guerra fjdiez@cbm.csic.es

Load the cells with calcein using Calcein-AM. Calcein leaks out with membrane damage. This is the basis for commercial live-dead assays using calcein in combination with propidium iodide. John Lemaster lemaste@musc.edu

In flow cytometry, many dyes such as PI are used as viability dyes. Examples include DAPI, 7-AAD, SYTOX, DRAQ7, etc. There are also fixable viability dyes from many manufacturers. And as mentioned by John, dyes such as Calcein-AM can be used as vital dyes to determine which cells are still metabolically active. One example of a publication mentioning this is: *Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition)* by Cossarizza et al., 2019 in *Eur. J. Immunol.* Chapter 3.4 "Dead cell exclusion, cell viability, and sample freezing" https://doi.org/10.1002/eji.201970107. Christian Kukat christian.kukat@age.mpg.de

FM dyes also work the same as PI and other DNA binding dyes but leaves nucleic acids untouched. Their fluorescence increases upon entering cells and binding intracellular membranes and the labeling intensity is proportional to the extent of damage. Here is our description of its use – https://doi.org/10.3791/51106. Jyoti Jaiswal jkjaiswal@cnmcresearch.org

I want to thank the many list members who replied both on and off list. We received many suggestions, including using calcium indicators, a variety of suggestions for anionic dyes that do not enter intact cells, many DNA indicators in case we do not like PI, references on the use of PI for this purpose, and references from people who have done similar studies. So, we have a lot to chew on, and I can say that just about anything I might do with a scope has been done by someone (or many) on this list. Aryeh Weiss aryeh@cc.huji.ac.il

Nuclear Marking and Segmentation

Confocal Listserver

DAPI and similar fluorophores are regularly used to highlight nuclei, but the staining is inhomogenous within nuclei and can vary substantially between nuclei in the same specimen. This makes segmentation for quantitative analysis difficult. So, any suggestions for a marker that produces a more uniform fluorescence within individual nuclei and less variation between nuclei, which would make image analysis simpler when we only want to know where the nuclei are. ***Jeremy Adler jeremy.adler@igp.uu.se***

Propidium iodide (PI) and To-Pro-3 have few 'details'. Broad-emission spectra should be spectrally (and FLIM) unmixable. I encourage our users to use Zombie NIR (BioLegend) both because it is way out in the near infrared and has a cool name. PicoGreen is also very interesting because it can be used at low concentration and 'lights up' mitochondrial nucleoids (in addition to nuclei) in live cells. A 2021 Nature Communications article (https://doi.org/10.1038/s41467-021-23019-w) published a new dye that they like over PicoGreen (synthesized by their lab). Anti-histone (or antibody to mCherry est for FP-histone H2B) is also a possibility. Segmentation: no DNA counterstain will solve the problem of closely apposed nuclei. I suggest ditching the DNA counterstain and moving to a nuclear pore complex (NPC) or nuclear lamina label. NPCs also provide discrete structures that either work as point-spread function targets or as resolution (or precision localization) test targets. It also helps to multiplex. There is no need to be stuck on DAPI, green, red, or NIR. For example, the Germain lab 2020 PNAS published a 65plex sample (up to 11plex per iteration) https://doi.org/10.1073/pnas.2018488117. George McNamara geomcnamara@earthlink.net

I second the recommendation of using a nuclear periphery stain for segmentation. If you're working with metazoan cells, laminA antibodies give a smoother signal than nuclear pores but either can be useful. Abby Dernburg afdernburg@berkeley.edu

We like to use laminA antibodies. They provide robust staining and are easy to segment. Sylvie Le Guyader sylvie.le.guyader@ki.se

Preservation of TdTomato Signal during Tissue Clearing

Confocal Listserver

We tried ethanol/ECi-based tissue clearing on whole mouse brains with endogenous TdTomato fluorescent signal. After the clearing, there was no TdTomato-specific signal left, but high autofluorescent background was present in the red fluorescent protein range. ECi cleared brain had some yellow color but was clear. Does anyone have experience with using ECi-based tissue clearing methods to clear mouse brain with endogenous TdTomato signals? Or any recommendations on which clearing methods will preserve endogenous TdTomato signal? In addition, what is the best method to reduce the autofluorescent background? Any suggestions would be greatly appreciated. Best, ***Tingting Gu tingting.gu-1@ou.edu***

Most often it is the dehydration step that causes the loss of fluorescence. Fluorescent proteins (FPs) require water molecules to maintain their confirmation. Once these are removed, the chromophore falls apart and fluorescence is lost. Methanol is the worst for this, the emission will be lost almost immediately. Ethanol is a little better and THF will give you a day or two of weak fluorescence. The final clearing solution (DBE, ECi, etc.) also plays a role, but the dehydration step does the most damage. The most recent solvent-based clearing protocols have found some tricks to better protect the FPs and preserve fluorescence for weeks or months. These include: 1) dehydration with 1-propanol or tert-butanol (or a mixture of both); 2) making sure all solutions have a basic pH (~9.0); 3) keeping the sample at 4°C during clearing; and 4) supplementing the solutions with Quadrol or Polyethylene Glycol. For more info see the following: uDISCO Pan et al., Nature Methods, 2016; a-uDISCO Li et al., Frontiers in Neuroanatomy, 2018; and PEGASOS Cell Research, 2018. Doug Richardson ds.richardson@gmail.com

We've maintained tdTomato expression in brain samples cleared using CLARITY at the Wyss center in Geneva. The signal is very bright after clearing. https://imgur.com/a/eNbqVEz; https://i.imgur.com/ewweZex.png?fb]<https://imgur.com/a/eNbqVEz; TdTomato CLARITY cleared mouse brain https://imgur.com/a/eNbqVEz. Sverre Grødem groedem.sverre@outlook.com

I second the advice from Doug and Sverre and I would also note that it is important to have a strong promoter with high expression if you want to preserve endogenous expression for clearing. Some protein loss is inevitable, so if you're starting with low levels and have high autofluorescence (which is common), things will be even harder. Most publications I've seen with good retention seem to use Thy-1 or a viral promoter. We have recently published some guidance for navigating these common difficulties with clearing and imaging experiments in a Nature Protocols tutorial-style review: https://doi.org/10.1038/s41596-021-00502-8. Since there are so many individual factors that go into any such experiment, we aim to give general advice and warnings, similar to the recent Jonkman confocal tutorial. Among other topics, we include notes on autofluorescence and why the yellowish hue in samples is evidence of protein retention (a good thing!) that shouldn't interfere with imaging. Hopefully you and others will find it helpful. Good luck! Kurt Weiss kweiss@morgridge.org

Immersion Fluid Options for Long Time-Lapse Imaging with Long Working Distance Water Objective

Confocal Listserver

I am demoing a long working distance water objective from Leica (HC FLUOTAR L 25x/0,95 W VISIR, WD: 2.4mm #506375) in an inverted configuration and am running into some challenges. While the objective is

very nice, the long working distance presents a problem for long duration time-lapse imaging with multiple positions. Water works fine, but it eventually evaporates (in hours). ZEISS Immersol W does not evaporate but cannot hold the column when the objective is over a mm from the coverslip (it drips/slides off). Leica has an automated water dispenser, but it is not available for my current demo. I was wondering if anyone had any ideas for other immersion options that have a surface tension and RI similar to water, but don't evaporate as easily. ***Pablo Ariel pablo_ariel@med.unc.edu***

We use Viscotears (artificial tear-fluid replacement) as the immersion fluid with exactly that lens (and its 10x and 20x relatives) for *in vivo* imaging of organoids transplanted to the anterior chamber of the eye with great results. This should be available from a pharmacy. Tilo Moede tilo.moede@ki.se

Many years ago, we used Suave hair gel. It worked for multiple positions fairly close together. Gary Laevsky glaevsky.lists@gmail.com

A few years ago, people on this listserv suggested jelly for sonograms and Genteal eye gel. Here is info on the latter http://microscopynotes.com/710/genteal/index.html Michael Cammer michael.cammer@med.nyu.edu

We have used Genteal Severe Gel (Hypromellose 0.3%) with our long working distance lens on an inverted system. It lasts overnight but will not last 48 hours without replenishment. For longer experiments, we re-apply during a break in imaging, roughly every 12 hours or so. We also made a little wall out of a glove finger to hold the gel in place and I think this reduces evaporation as well (but it limits ability to do large Z-stacks as the glove material folds under pressure). Theresa Swayne tcs6@cumc.columbia.edu

Why not use the Cargille oils that match the refractive index of water? You can get separate oils for matching water at room temperature (~27°C) or incubation temperature (37°C). I use these for long-term imaging without any evaporation issues. Timothy Feinstein tnf8@pitt.edu

ZEISS sells oil with a refractive index of 1.33. This will go with any water immersion lenses. We are using this for all of our microscopy systems for extended time-lapse imaging. Ammasi Periasamy ap3t@virginia.edu

Long working distances require that the fluid maintain its structure, such as with high viscosity or strong surface tension. The oils run down the side of the lens. Michael Cammer michael.cammer@med.nyu.edu

Thanks all for the many suggestions. I will try the eyedrop approach and let everyone know if we come up with some sort of containment that can reduce evaporation further. Pablo Ariel pablo_ariel@med.unc.edu

Uniform Focus Across a Field of View

Confocal Listserver

We have a question about tilt of a specimen or detectors leading to shift of focus across a field of view. We have used many confocal microscopes over the years and have not had problems with the image being in focus corner-to-corner across the field of view when using

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planapochromat lenses. This includes newer microscopes with wider fields of view and a spinning disk confocal with a Cairn twin camera setup. (The cameras, however, required substantial alignment to attain this). We use standard and unusual stage inserts and a variety of samples and are able to focus across the entire field. This includes imaging in reflection mode, TIRF, Nomarski, epifluorescence, and fluorescent confocal. We have a new microscope, however, which does not have focus uniformity. I believe it is a dual camera alignment issue and we have been discussing this for almost a year as an installation issue, but recently the manufacturer has insisted that the only way to fix the problem is to use screws on the stage to adjust the tilt. I am concerned that this is going to be a major problem in a core facility with users who will play with anything. It is going to add substantial burden on staff. Also, it means that standard inserts will not work. And it makes me wonder what this will do to the PSF if the coverglass isn't perpendicular to the light path. Have we been unusually lucky all these years to never have encountered this problem before? Are we unreasonable to expect the microscope to be installed with all planes parallel for routine use? Is this type of stage alignment standard acceptable procedure in other labs? Thank you. ***Michael Cammer michael.cammer@med.nyu.edu***

As a former ZEISS affiliate service tech, back when I was young, and this still applies today, we used a stage square to make sure that the flat surface of the nosepiece and the surface of the stage were parallel with each other. The device was made on a lathe. The base was about 24 mm diameter and screwed into the nosepiece and extended 45 mm to a flat disk about 75 mm round. I would carefully lower the 75 mm disk to the stage surface to make sure it touched evenly. This can be used on uprights or inverts. This enabled the user to check for parallelism issues caused by either the stage mount or the detent in the nosepiece. If the detent is worn it may cause a rotational misalignment which causes the focus to be nonuniform across the field. It is also best to have a crosshair objective to check alignment of the optical axis of the scope. Check with your scope manufacturer to see if they can or will provide these reference parts. Dan Focht dan@bioptechs.com

We have a dozen confocals and none of them have any appreciable focus shift across a single field of view. In fact, on the inverted microscopes with a thin sample, if a user has a paper label close to one edge of the slide and no label on the other, this should cause an obvious tilt as the slide sits higher on one side than the other, but from a single field of view it isn't very obvious unless you're looking for it. The effect of the label becomes obvious if you move the stage as the sample quickly goes out of focus as you move side-to-side. If there is a substantial tilt across the field of view, I would agree that the manufacturer should address this, and not with stage inserts: we usually remove the stage insert tilt screws as they tend to jiggle their way down with gravity and end up misaligning the stage tilt more than anything else. Maybe you want to create a shared cloud folder (Dropbox, OneDrive) and drop an image or 2 in there so that we can see how bad it is? If it would help, I would be willing to take some images across several of our confocals and upload them to the same folder. I'm thinking a short z-stack of a thin sample (Molecular Probes Prepared Slide #1, BPAE cells for example) would illustrate the problem nicely. And maybe a second test would be to focus the same slide in the middle of the field of view, then take a tiled image (say, 5 x 5). Choose an objective and we can try to match it. Anyone else interested? James Jonkman james.jonkman@uhnresearch.ca

As a follow-up to our previous conversation: This microscope (not a confocal) comes standard with a unique levelling stage insert. It has X and Y adjustment screws in opposite corners and a pivot point in the other corner. This stage insert can correct for any tilt throughout the system, although it can take a long time to get it right. Since you mentioned TIRF, I assume you're not using that insert, but a live-cell heated stage insert. I do not like the standard heated stage that ships with that system. In our facility it eats objectives. Therefore, we ordered the PeCon push and click system for live cell holders (and rely on the plastic incubation box plus time to maintain temperature). We had to sign a waiver acknowledging that this insert was not approved for use with the system and that we may experience unwanted thermal drift and uncorrectable tilt. It turns out we didn't see any of this and now exclusively use the push and click system for live and fixed samples as it can also hold slides. The adjustable stage now collects dust in a box and our images look great. No need for adjusting stage screws. To be fair, we don't do TIRF, but we image 200 µm beads at every SIM session, and these are always in focus across the field. It was very difficult to achieve similar uniformity with the adjustable stage without a good 30–45 mins of adjustments. Doug Richardson ds.richardson@gmail.com

For our ZEISS confocal with Airyscan we have a leveling stage insert that came with a protocol for using it. If you want to spend a few minutes you can get a small field of view very flat. Some stage inserts are fairly uniform across vendors so perhaps this could be adapted to your system. Brian Armstrong barmstrong@coh.org

Does your microscope have eyepieces? If so, checking for a flat field with the eyepieces should solve the issue whether the tilt comes from the camera alignment or from the stage. Beads on a glass surface or any other thin sample should be a good sample for this. Also, how does the tilt behave with different objectives, say 40x and 100x? You should get a different outcome depending on whether the problems arise from the sample/stage or behind the intermediate image. Steffen Dietzel lists@dietzellab.de

Or (seconding to Steffen), does the microscope stand have another pot where you could connect a camera directly? As already mentioned, if your sample does not go too much out of focus when you move the stage a long distance (1 cm) then you can't fix your issue with stage insert leveling screws. The issue then must be somewhere between the objective lens and the camera (filters in the main turret are not suspect, as these are in transmission and in infinity space; thanks Dan for pointing out the nosepiece). Alternatively, the whole XY stage is not bolted correctly to the microscope frame. Zdenek Svindrych zdedenn@gmail.com

Beam Pulsing Issues

Microscopy Listserver

On our Philips CM12 TEM, the beam is pulsing in SA mode, the technician has changed the filament and checked the HT voltage. This helped but did not eliminate the pulsing. We do not know why this issue is occurring and the technician has not been able to fix it properly. Has anyone encountered this problem and if so, what do we need to check or replace? Thank you in advance. ***Sara Rizzo sararizzo125@gmail.com***

You do not mention if it's a very regular (an electrical issue) or irregular pulsing (charging, including vacuum leakage). Only in the SA mode? That sounds like a charging issue. Clean the SA aperture holder and apertures. It could also be a bad relay for switching to diffraction. This would focus/defocus which would result in beam pulsing. Richard Edelmann edelmare@miamioh.edu

There are several possibilities. Abrupt changes in visual screen brightness might be caused by charging, arcing, too high emission current (not necessarily high setting), electronics issue, etc. Vitaly Feingold vitaly@sia-cam.com

When was the last time you cleaned or replaced the apertures in the column? One can replace them or clean them in a plasma asher. Usually, it is cheaper to replace them. John Minter jrminter@gmail.com

Steve Chapman of Protrain in the UK has responded in the past to similar pulsing issues. In those instances, Steve's vast experience pointed to a dirty column. When the column gets dirty, little arcs between the main beam and the ground side of the column are common. Maybe, after you exhaust all other possibilities, you should open the column and clean it. In SA mode only, when the objective lens is on, pulsing suggests the issue could be dirt and or fibers around the objective lens area. Pete Eschbach peter.eschbach@oregonstate.edu

Thank you for your help. Sorry I did not mention that by SA mode, I did not mean selected-area electron diffraction. On FEI instruments, the SA imaging mode is when the magnification is higher than 5600X or 6200X. We have a beam pulsing issue in both imaging and diffraction modes. The FEI technician changed the filament, checked out the HT circuit, and replaced the 30 V double-deflection coil. I checked it out today and there is beam pulsing at 60 kV, 80 kV, and 100 kV at magnifications higher than 2650X. I can't see pulsing at low magnifications. There was irregular beam pulsing last month, but now it is regular pulsing. The condenser apertures, objective apertures, and SA aperture were replaced a month ago. Thank you. Sara Rizzo sararizzo125@gmail.com

Can you qualify your description of pulsing a bit better? Does the entire image appear to be pulsing or only the beam? Use a holey carbon film and alternately spread the beam over a larger area covering multiple "holes" versus focused within a single hole. Is the effect and magnitude the same? Nestor Zaluzec zaluzec@anl.gov

As people have already mentioned, there can be a number of causes for pulsing. If the beam intensity is flickering but the image is stable, there is likely charging/discharging on the Wehnelt cap. Basically, it is changing the "emission" setting of the gun by affecting the bias voltage. If you see the image size pulsing, it suggests that there is HT instability. When there is an arc in the gun, the HT will momentarily drop. The lower-energy electrons will be deflected more by the lenses, and you will see the image rapidly expand then shrink back to its original size. If you see a stable image but the beam diameter fluctuates, there is current instability in the C2 lens. If the image magnification fluctuates, the instability is most likely in one of the imaging lenses. An instability in the diffraction lens will cause the objective aperture to change focus in diffraction mode and the SA aperture to change focus in image mode. Henk Colijn colijn.1@osu.edu

If low magnification is stable and the problem only appears in SA mode, then the problem is not likely to be gun or HT hardware/circuitry related. It is not likely caused by contamination or charging either. It is likely to be an electronics issue. Henk explained general symptoms completely. Troubleshooting is required. By "30 V double-deflection coil" - do you mean a 30 V doubler unit on the left side of the power supply cabinet? Perhaps a silly question - is the wobbler ON? Is any alignment mode ON? (There are several such modes). Vitaly Feingold vitaly@sia-cam.com

Vitaly, sorry for my mistake, I meant a 30 V doubler unit. Neither wobbler nor alignments are ON. Thank you, everyone. I will show your comments to our technician, hopefully, he can fix it the next time. I will keep you updated. I have uploaded a video of beam pulsing here: https://www.mediafire.com/file/9nfnix4ck23jozv/beam+pulsing.mp4/file Thank you again. Sara Rizzo sararizzo125@gmail.com

In the video I saw the magnification changing by a few % - is this the symptom? (Your camera was moving so it was hard to tell). If yes, then either the HT isn't stable, or the lens(es) current(s) isn't stable. There was one big jump in diffraction mode—was this also a symptom? On a general note - when recording a video, please fix the camera (smartphone or whatever) in space so the camera itself doesn't move. Thankfully it was possible to reference image to circles on the phosphor screen, but next time rest your smartphone against a wall or TEM, so the field-of-view won't move. Are the symptoms what I described above? Vitaly Feingold vitaly@sia-cam.com

Sorry about that, I recorded new videos with a fixed camera recorded at mag 5600 X, spot size 3 and beam pulsing with no sample: https://www.mediafire.com/file/61o25rfa319ywjp/beam+pusling+no+sample.mp4/file Beam pulsing with sample: https://www.mediafire.com/file/bzvizngisfevl4q/beam+pulsing+with+sample.mp4/file The beam is pulsing in and out. Thank you. Sara Rizzo sararizzo125@gmail.com

Looks like a discharge in the gun. Possibly the Wehnelt. A look at the caustic spot might glean some insight. Regards, Jaap Brink jbrink@jeol.com

Arcing in the gun HT to ground would cause a way more dramatic effect. Your videos don't show that. Extra conductivity in the gun or HT cable (to ground) once present usually goes only one way, not back and forth as in your videos. Arcing or extra conductivity in the gun cathode-to-Wehnelt could be somewhat similar to your videos except it would cause obvious brightness/emission changes. Your videos don't show that either. Same as before - lens(es) current(s) or HT isn't stable. Vitaly Feingold vitaly@sia-cam.com

Wonderful videos, and really very useful in diagnosing the issue. Ok, so here is my best guess first thing to look at, the lens reference resistors. Both the C2 and the OBJ lens ones. The CM12 is based on the EM420 and after several years the lens reference resistors that compare the current in the resistor to the lens go bad. It is like a Wheatstone bridge circuit and the lens voltage/current is compared to the balanced power resistor for stability, when they go bad. They act as a LR circuit and pulse slowly as they fail. I would have your tech spray with a cryogen and check. If you freeze them they stabilize, and then fail when they warm up. Let us all know how it goes. It is now like a game to the community. David Bell dcb@seas.harvard.edu

I hadn't thought of the lens measuring resistors (aka "foot" resistors). However, now that you mention it, that sounds like a likely source of the instability. I seem to remember that they were on a water cooled heatsink. I'll put my vote in for your suggestion! Henk Colijn colijn.1@osu.edu

The thermometer connected to the scope shows 68–70°F. There are other scopes in the room, all of them connected to one chiller, none of them have this issue. Sara Rizzo sararizzo125@gmail.com

What do you think about a leakage current from the lens coil to the lens housing? This may not occur very often but is easy to measure. You only need an insulation tester: Unplug the lens coil and connect the tester at one of the lens coil contacts and at a cooling tube or water connector of the lens. (Do not disconnect water!) Bad insulation from the coil to housing will cause a leakage current which will deteriorate the stability of the lens current. If the insulation is bad, you need a new coil. Winfried P. Send winfried.send@gmail.com

Generally correct. Devil is in details. Firstly, it is not necessarily a lens coil. Diagnosing this mode of failure is a highly test-condition-dependent.

If the tool is an ohmmeter, then [some of] the conditions are voltage of the ohmmeter, duration of the measurement cycle, air humidity, degree of mechanical disturbance of the component being tested (like moving, vibration). The intermittent nature of the problem is the direct result of such dependencies. One must make an educated guess and then give it a try. If "educated guesses" consistently miss the root cause then it becomes an endless affair with an erroneous verdict in the end: "instrument is worn-out and needs to be replaced" or "spare components no longer available" or similar. Usually not true. Old instruments can be maintained reliably and indefinitely. Besides, the CM12 is fairly modern. Modern design, modern composition, modern component base. Components can be easily substituted or repaired/fabricated, with very few exceptions if any. Vitaly Feingold vitaly@sia-cam.com

Update on our scope. Our technician replaced the Wehnelt insert cap and HT cable, the beam is not pulsing anymore. I was working with the scope all day and everything was fine. I will keep you posted if the issue comes back again. I would like to thank you all for taking the time to share your valuable knowledge and experience with me. I appreciate your help so much. Best wishes, Sara Rizzo sararizzo125@gmail.com

That is good to hear. Were you using a LaB_6 or Tungsten filament? I used to work for a company that designed, developed, and built cathodes and electron gun modules for thermionic sources; mainly LaB_6 and CeB_6. When the cathode reached the end of life the customer would send their gun assembly back and we would do a failure analysis report before we replaced the cathode and refurbished the Wehnelt. I always observed a deposited film that had a composition of LaB_1-xOx. Eventually the film would begin to delaminate, and smaller flakes would curl up towards the cathode. At the time we hypothesized that there was charging and dis-charging of these flakes during operation. Essentially, the film/flakes were acting as a dielectric layer and after the charge reached a certain threshold the film would discharge, and the cycle would repeat. This changes the electric field lines near the entrance of the Wehnelt, which modulate the beam current (image intensity). Andrew Thron athron@ucdavis.edu

Horizontal Lines Occur During Image Capture

Microscopy Listserver

I have a Hitachi SU1510 and have been experiencing image degradation on slow scan or capture, usually exemplified as horizontal lines during the scan. I have replaced the filament and completed an aperture alignment as required but the problem remains. Any ideas? ***Harry Murray harry.murray@dfo-mpo.gc***

Usually this type of artifact is in the detector, not the beam itself. From my experience it's more common in SSD-type detectors, but I've seen it with end-of-life ETD or ICE-type detectors as well. Do you have the same artifact in multiple SE detectors? At least in FEI tools, the artifact can also be caused by a bad preamp. Kajir Mellor kajirmellor@gmail.com

This is one of those times it would be nice to post a link to a picture. My first thought might be charging in the sample, but detector electronics are certainly another likely candidate. It is also good to check the other detectors, for example, BSE versus SE or ETD at different collection voltages. Warren Straszheim wesaia@iastate.edu

Something similar happened to me on our Scios Dual Beam FIB. Our trinity detectors - a set of below-the-lens, in-lens, and above-the-lens detectors - started showing lines in the images that did not match the contrast of the overall image. The images were also noisier than usual. The issue ended up being a pre-amplifier. Andrew Thron athron@ucdavis.edu

STEM Settings

Microscopy Listserver

As my TEM is down, I have been working on alternative instruments. I have a ZEISS NVision 40 with a STEM option but no instructions. I can insert the detector and align the specimens, but that is as far as it goes. Does anyone have suggestions for the correct settings for: 1) operating voltage? 2) Which aperture? 3) What settings for the three "quadrants" of the detector. Any information would be much appreciated. I can get an image but it's not that great as I'm guessing at the above settings. Thanks in advance. ***Emma S. Perry emma.perry@maine.edu***

Since you have an image, I'm assuming the sample is thin enough for transmission and you have the sample mounted in the appropriate STEM carrier. You may need to adjust the sample-to-detector distance by moving the stage up or down, but do this carefully. Generally, higher accelerating voltages will give you a smaller probe and thus higher spatial resolution. You should also work at the maximum voltage to ensure maximum sample transmission, unless your sample is very thin. I would start with a large probe and aperture to make sure you can find the sample and optimize the image, then you can move to smaller spots to increase the spatial resolution if needed. I'm not familiar with ZEISS STEM detectors, but you should enable all the (HA)ADF and/or BF diodes to maximize the signal collected. Keep in mind the sample-to-detector distance will determine where the signal is landing on the detector. If you still can't get anything reasonable, be sure to inspect the STEM detector carefully. In a shared-user facility, STEM detectors occasionally get crashed into stages or scratched/broken from unsafe insertions. Check the diodes carefully to make sure they aren't scratched or broken and check the wiring harness to make sure it's intact. Since you have something of an image, hopefully all is okay with the detector. Chris Winkler crwinkler@ncsu.edu

MT

Crossword Puzzle Answers

See puzzle on page 66.

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