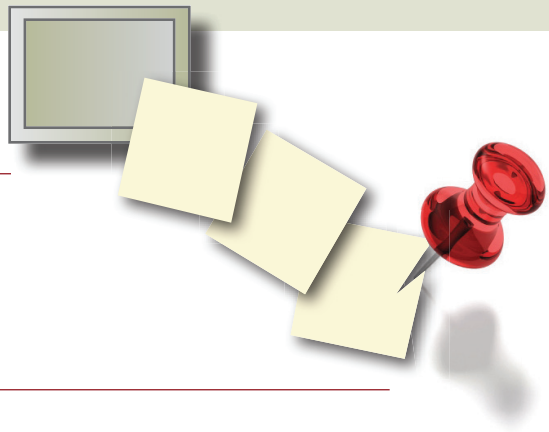
NetNotes

Edited by Bob Price
University of South Carolina School of Medicine

Bob.Price@uscmed.sc.edu



Picric Acid Disposal

Microscopy Listserver

Good afternoon, we have found a bottle of old picric acid, which someone may have been using for microscopy purposes a long time ago. Does somebody have a suggestion for disposal? Best regards, ***Antonio D. Molina-Garcia antoniom@ictan.csic.es***

Around here, the university calls the local police department bomb squad and shuts down the building until they dispose of the bottle. Literally and seriously. This has happened here. So, check with your organization's hazardous waste people before even moving the bottle. There may be legal issues you have to face. Phil Oshel oshel1pe@cmich.edu

Same thing happened at one of my labs. During an inventory, Safety found a 1lb jar of the stuff with a cracked lid. The bomb squad shut down the building, took it out back (we were on the edge of town), and blew it up with C4. There was quite a thump when it was detonated. Tons of ensuing paperwork and chastisement for the lab supervisor. Becky Holdford pfadiva@gmail.com

Here is a link that you might find helpful, "Safe Handling of Picric Acid". https://ehs.wisc.edu/wp-content/uploads/sites/1408/2020/08/CHM-GUI-006-NEW.pdf. Bob Carter bobwcarter@gmail.com

Out of curiosity, I looked it up. Picric acid is a high explosive, slightly more powerful weight-for-weight than TNT. It was formerly used as the main charge in anti-tank rounds. I thought it was a primer only. A pound is a terrifying amount. Good thing whoever found it knew what they were looking at. Rick Mott rmott@pulsetor.com

It is also a contact explosive if it dries out. Hence the horror at the cracked lid. In the distant past, some poor soul had the job of making sure it was kept damp. One pound is an extravagant amount; whoever ordered it originally made sure they had enough for an entire career. It was used ages ago as a component as a metallurgical etchant, but I was in a lab for 10 years and never once saw it used or even referenced. Becky Holdford pfadiva@gmail.com

My 2 cents:

1. The simple approach. If you have it dry - keep it dry. If you have it wet - keep it wet. And keep it away from metals. I have a nice photomicrograph of grain boundary migration of picric acid coming out soon https://www.researchgate.net/publication/359865616_pH2_Postcard_2021_65_by_9_R3_Sec.
2. From George Vander Voort: Picric Acid - Hazards and Safe Usage:

Picric acid (2,4,6-trinitrophenol, $[(NO_2)3C_6H_2OH]$) is widely used in metallography labs for common steel etchants known as picral, a 4% solution in ethanol; Vilella's reagent, 1 g picric acid and 5 mL HCl and 100 mL ethanol; and alkaline sodium picrate (2 g picric acid, 20 g NaOH, 100 mL water), for coloring M3C and M6C carbides, as well as several other formulations. Picric acid was formulated by Peter Woulfe, a British chemist, in 1771, although Glauber is claimed to have written about it in 1742. The name comes from the Greek word pikros which means bitter, as picric acid has a bitter taste (it is toxic). Initially it was used to dye fabrics yellow. In the early 20th century, workers producing picric acid were sometimes called canaries, because their skin became stained yellow. The explosive nature of picric acid was discovered in 1885 in England, which led to the 1888 development of an explosive called Lyddite, named after the location of the studies, Lydd, England. Another source states that the explosive nature of picric acid was first known in 1830, and it was proven to detonate in 1873. In 1894, Russian scientists manufactured artillery shells using picric acid salts, and picric acid-based explosives were used in WWI. Anhydrous picric acid is related to TNT, a much more potent explosive. When concentrated, it will attack metals, producing shock-sensitive salts that are explosive. This was discovered in 1916 at a French ammunition factory when a fire caused molten picric acid to wet a concrete floor, forming calcium picrate, which detonated killing 170 people. Fortunately, there have been no documented cases of explosions from picric acid in laboratories, according to Phifer [1].

If it is wet with water, it is not an explosive hazard and any attempt to blow it up by a bomb squad will only result in picric acid being spread all over the immediate area. The concern has always been in finding an old bottle that has dried up producing dehydrated picric acid, and if it has a metal cap, rather than a plastic cap. In such a case, shock-sensitive metallic picrates may have formed at the cap-bottle interface. The solution is to have a robot pick it up and re-hydrate the picric acid after opening the bottle under water. If the cap is plastic, and the acid has dried out, friction from opening the cap could cause detonation. The solution here is to place the bottle in a large bucket or tank of water and allow water to dissolve any dried picric acid on the cap threads. Leave the bottle in the water for a few days until some water can be seen inside the bottle. Then, while under water, open the lid and re-hydrate the picric acid.

Obviously, the wise lab manager checks the picric acid bottle periodically (which can vary with lab usage of picric in etchants) to make sure that the picric acid remains wet. Today, bottles are sold with at least 30% water content. A good practice is to keep a log of when the bottle is checked and when water is added. Also, use only plastic or glass spatulas to remove picric from the bottle and add it to the etchant. Do not use metal spatulas and clean the cap and threads on the bottle and on the cap with a wet paper towel. If plumbing consists of copper piping, do not dispose of picric acid by pouring it down the drain as explosive metallic salts could form.

Virtually all chemicals and solvents used in the laboratory are dangerous; hence we must develop good safe laboratory practices and teach our employees what to do to avoid problems. Personally, I have never heard of a problem in a metallography/materialography laboratory from picric acid but, I know of four accidents from nital (2–3% nitric acid in ethanol; one accident used isopropyl alcohol instead), which people consider to be very safe to use. Every dangerous chemical or solvent cannot be outlawed for use, or we will not be able to work. Even water can be considered as being dangerous because every year many people drown, but we would never consider outlawing water. The solution is to establish a good laboratory safety program and train employees to develop safe working habits. Vander Voort [2–4] has summarized lab safety aspects as a sequel to the superb treatise by Anderson [5]. ASTM E 2014 lists a number of books on laboratory safety and is a good source of information on metallography lab safety.

[1] R Phifer, *Picric Acid: When is Panic Justified?* Speaking of Safety, 9(2) (2000), 1–3.

[2] GF Vander Voort, *Metallography: Principles and Practice* (1999) 148–159, ASM International, Materials Park, OH.

[3] RC Nester and GF Vander Voort, *Safety in the Metallographic Laboratory*, ASTM Standardization News (20) May 1992, 34–39.

[4] GF Vander Voort, *Laboratory Safety in Metallography: Metallography and Microstructures*, 9 (2004) 1081–1089, ASM Handbook series, ASM International, Materials Park, OH.

[5] RL Anderson, *Safety in the Metallographic Laboratory*, Westinghouse Res Lab Sci Paper No. 65-1P30-METLL-P2, March 20, 1965.

[6] ASTM E 2014-99 (2005), *Standard Guide on Metallographic Laboratory Safety*.

Andrew Havics ph2@sprynet.com

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Years ago I managed a histology core that had inherited chemicals from other labs. Our risk management people carefully removed and disposed of the three one-pound plastic jars of picric acid (still wet, but the plastic containers were deteriorating) on a weekend. I spoke about this particular incident with an elderly faculty member who had been a beachmaster with the US Marines in the Pacific during WWII. He said that the torpedoes used by naval aircraft in WWII used picric acid as the explosive (chemically it has some similarities with TNT) and it was notoriously unstable. He recalled a landing craft that was shuttling a load of torpedoes from a larger ship to a captured island airbase. The load shifted when the landing craft was out in the water and there was nothing left of the landing craft. Histology labs use picric acid as a mordant for staining and it is an ingredient in Bouin's fixative, a favorite for investigators of ovarian tissue. Doug Cromey dcromey@arizona.edu

My high school chem lab found a dried-out jar hidden away. They called the school district, who sent a person out in a rickety old truck. He put the jar in a cushioned box at the back of the truck bed and drove away taking care to go really slow over the potholes in our parking lot. Yep, ya can't make this stuff up. Kevin McIlwrath kmcilwrath@jeol.com

Sounds like fun! We still use picric acid ("yellow fix") as part of the fixative mixture for some vet med biopsy preps. In an attempt to avoid environmental safety crews' panic attacks, check for any crusty deposits on the cap area. Hopefully the chemical is in a poly bottle rather than metal and there is still liquid inside. I have stories of finding old ether cans hidden in an adobe wall of a hospital building used at one time for storage and converted to lab space. Ah, fun times. John Shields johnshields59@gmail.com

I have kept a 100g bottle of picric acid that I "inherited" from a lab that moved from NYC to NJ. It could not be moved across state borders. The investigator I got it from had it for about 10 years and I've had it for close to 20 years. I keep it topped off with deionized water and use the saturated aqueous solution in our primary fix according to Ito and Karnovsky (Formaldehyde-Glutaraldehyde Fixatives Containing Trinitro Compounds *J Cell Biol* 39 (1968) Abst.418. It really helps with membranes (I got it from an "eye" lab that studied the outer rod segments). It took a lot of careful discussions with our EHS people to finally assure them that I would ALWAYS keep it wet, wipe the lip of the jar to prevent crystal formation, etc. It is great stuff when handled with the care it demands. Lee Cohen-Gould lcgould@med.cornell.edu

Many thanks to the many people that sent helpful and informative comments. We will contact the relevant authorities (explosive experts) for the disposal of this product. Thanks again. Antonio D. Molina García antoniom@ictan.csic.es

This thread has been a real blast. Oops, sorry, couldn't pass it up. Jonathan Krupp jkrupp267@gmail.com

External Light Source

Confocal Listserver

Hello everyone, I am having an issue with a Leica EL6000 external light source. We use it for looking at fluorescence through the eyepiece of a Quorum Wave FX Spinning Disc system. Lately, when I turn it on, the lamp stays on for about 15–20 seconds, then shuts off (the lights on the switch and shutter indicator stay on, but the mercury metal halide bulb inside shuts off). If I switch it off then back on again, the lamp turns on but then shuts off after a little while. When I open the lamp house, there are small white deposits all over the bulb, notably around the connector and where the metal reflector connects to the plastic casing. I've removed most of it with a toothbrush and have cleaned the connectors, but the issue still persists. I was wondering if any of you might know what these deposits are, and if you think they are the cause of the issue. It could be that it is time to replace the bulb, an issue with the power supply, or something else. So, I wanted to see if anyone has any ideas that I might try before moving forward. Thank you, ***Mathew Duguay mathew.duguay@ladydavis.ca***

How long has the lamp been used? Do users respect the restrike/cool-down time? Is there any white deposit inside the bulb? I don't remember which bulb the EL6000 uses, but if there's a milky-white coating inside the discharge tube, it could be from devitrification of quartz. William Giang wgiang@pennstatehealth.psu.edu

I often see this whitish residue when changing these types of bulbs, both with the Leica EL6000 and the various XCite lamps. I never see the residue on the actual bulb surface though. It is usually just in the bottom of the lamp house and sometimes on the base of the lamp. This is quite normal. How many hours of use has the lamp accrued? Mercury metal halide lamps should be changed at 2000 hours. The other question I have is whether the lamp is operated with a minimum run time. It is not an LED, so I recommend at least 30 minutes of use before the lamp is turned off and a 30-minute cool-down period. In fact, in our facility, we have a mandatory 1 hour on, 1 hour off rule, and if the microscope is to be used within 2 hours or less, we do not turn it off. I believe this helps with ensuring good lifetime/stability for the bulb and also for safety, as it does contain mercury. Some light sources will not allow the lamp to be turned on again if it is still hot (within a certain period). The fact that the bulb is turning off might also be a problem with the power supply, or perhaps the bulb is not seated properly or it is overheating. I know it sounds basic, but I would also check that all power cords are properly inserted. Finally, if there is residue on the bulb surface, I would replace it. Although the bulbs are expensive, you are losing valuable time and there could be a safety issue. Jacqui Ross jacqui.ross@aucland.ac.nz

We don't have a Leica light source but have observed something similar with XCite and Nikon light sources. Sometimes overheating of the lamp will occur if the fan vents are obstructed. Konstantín Levitskiy microscopia-ibis@us.es

Thank you for your replies. The bulb predates my time here (over a year) and the screen indicating lamp use doesn't work, so it's impossible to know how many hours it's been used for. I will check things that some of you have mentioned, as well as replace the bulb if the rest seems fine (rather than retire it completely). Mathew Duguay mathew.duguay@ladydavis.ca

Given the unknown number of hours of use, I would replace the bulb. I use a mini-vacuum cleaner to clean out the lamp house when I change the bulb. Jacqui Ross jacqui.ross@aucland.ac.nz

Broadband Femtosecond Laser for Multiphoton Microscopy

Confocal Listserver

Anyone out there with an opinion on use of the Fyla broadband femtosecond laser for multiphoton microscopy (https://www.fyla.com/product/sch/)? I am wondering if this is the way the market will go considering the cost of purchasing and maintaining Ti:Sapph tunable lasers. All the best, ***Peter Owens peter.owens@nuigalway.ie***

Hi Peter, I've worked with ultrabroadband systems like this before (8fs Menlo), and while they are good for certain applications, they add a great deal of complexity to a system. After extensive "playing" I concluded these systems are only worth it if performing advanced techniques such as coherent control.

Potential issues:

- Ultrabroadband pulses disperse greatly, and usually in a nonlinear fashion, so pulse fragmentation can be an issue.
- Due to dispersion, a spatial light modulator (phase) system is required to keep the pulse near the temporal width it possessed directly out of the laser.
- The dispersion of the microscope on such a broadband pulse is very high, so often a 2-stage compression system is necessary to compensate for the group velocity dispersion/group delay dispersion (GVD/GDD).
- Broader-band lasers exist (I've worked with a system with nearly 300nm BW), but dispersion control becomes increasingly complex as spectral bandwidth increases.
- The power is distributed over the entire spectrum of the pulse, so without optimal dispersion control there is almost no signal.
- If pulses are compressed down to their minimum at the sample, the pulse is so short multiphoton phototoxic effects are a risk. I found my 8fs 85Mhz pulse laser bleached everything pretty rapidly.

Advantages:

- If the laser provides enough spectral power density, multicolor 2P with broadly separated dyes can be performed. A normal 2P system can do this for some dyes, but ultrabroadband lasers let you take this further.
- The two photons do not need to be the same wavelength; with good dispersion control, nearly the entire pulse can contribute to signal generation. A photon that is too red meets a photon that is too blue, and the pair add up to the energy you need to excite the fluorophore cross correlation (2P).
- If using a sufficiently sophisticated dispersion control scheme, a computer science student can be hired to use algorithmic approaches to manipulate the phase of the pulse components for coherent control of the fluorescence. This allows "hitting" the fluorophore in a way that is optimized in wavelength and phase to get the best signal, and to even toggle different fluorophores on or off to minimize bleaching and phototoxic effects, etc., by guiding the energy through the system via phase control.
- Very short pulses are good for other nonlinear effects, CARS, SHG, XFG, etc., and this can also be tweaked through coherent control of the pulse.

So, in short, investment in tight control of the dispersion and machine learning can get amazing imaging and some neat tricks. Otherwise, the extra complexity is much, much more trouble than it is worth. As a general comment, my money is on lower-repetition-rate, higher pulse energy systems. These typically have repetition rates in the 5–10MHz range, so performing fast video rate imaging with them is not possible, but the high energy-per-pulse yet low *average* power allows very deep imaging, which is the best use-case for 3P/2P in the first place.

For video rate, the Ti:Sapph still continues to give the most bang-for-the-buck, although wavelength extension systems that push out to 1300nm (for 3P) are very useful as well. Some of these extended wavelength sources are also low-repetition and these are common for deep 3P imaging at 1200–1300nm. Craig Brideau craig.brideau@gmail.com

I haven't used the SCH, so the below is pure speculation (as usual). I've worked with Ti:Sapphs a lot, and we mostly use the 700–900 nm range (things like NADH, GFP, etc.). Of course, longer wavelengths are great for three-photon and orange fluorophores, but the Ti:Sapph dies out at 1000 nm. Power is important. While we often used 1% of the available power, in some cases of deep imaging tens of milliwatts out of the lens may be needed. 15 fs pulses are not an advantage. With this huge bandwidth, even slight dispersion will lead to longer pulses than the standard "narrow band" 100–200 fs. And the offered range of GVD compensation seems quite narrow. The spectrum is centered around 1064 nm. Remember, with the logarithmic plot in the datasheet, every 10 dB is a factor of 10 power difference! With the very broad spectrum it's hard to predict what will happen to the pulse length if part of the spectrum is removed with a bandpass filter. A lot of power will be lost for sure.

In summary, the SCH is not a tunable laser, so it's hard to compare with a tunable Ti:Sapph. It might work OK in a home-built single-purpose system, but I wouldn't bet on it if connected to a commercial point-scanning confocal microscope. No commercial interest, but I remember Thorlabs' tunable and broadband femtosecond lasers were quite competitive (read: cheaper than Coherent). Zdenek Svindrych zdedenn@gmail.com

I think it would be a hard laser to use in a general purpose system. The pulse length is too short which will make setting the dispersion compensation precisely important, and for best results you may actually need to correct for both GVD and third order dispersion (TOD). At the same time the power output is low, which will make efficient use of power critical, which is hard if you need to add (lossy) TOD correction. Finally, since the bandwidth is 200nm, all optics will have to be extremely well achromatized (not just coated) for that (fairly unusual) wavelength. Using normal IR coated (but visible achromatized) objectives will give 950nm light focused tens or even hundreds of microns away from where 1150nm light focuses, which will not work. Similarly, the scanner optics will need to be extremely well corrected, especially for lateral color. All of this is doable, but hopefully you'd be designing the microscope around that laser and not expecting the laser to work with an existing microscope. One other general question I have is the odd spectrum, presumably generated from nonlinear fiber interaction. I see a spec for long term output stability (3 hr), but not one for noise. If this is a compressed nonlinear fiber output, the shot-to-shot spectrum can be noisy, which is fine for low-speed imaging (averaging thousands of pulses) but might be a problem if looking at resonant scanning (averaging a few pulses). Mike Giacomelli mgiacome@ur.rochester.edu

These comments are very helpful. I would like to continue this discussion, and to change the direction to include the current fixed line fs lasers on the market. What is the opinion on using fixed line lasers such as the Axon range from Coherent in place of something like the Incite X3 or Chameleon tunable lasers? (that is, using a 780, 920 and a 1040/1064 nm set of fixed line lasers that would cover the absorption cross-section of most common fluorophores). Again, the emphasis here is on the maintenance and lifetime of the light source. It would be great to see what those already using MP systems think about this, and their wish list for an MP system to be used in a core facility. Peter Owens peter.owens@nuigalway.ie

That is something I have thought about. My experience with 1040nm Ytterbium fiber lasers has been positive. We have two from Menlo and they work extremely well. You push a button and 3 seconds later they're mode locked at 2.5W. We have even put them in mobile cart systems, bumped them into elevators, driven them to other sites, and they still work normally. Conversely, our previous Coherent Chameleon had trouble over its entire life despite repeated service from Coherent. Not sure if we just got a lemon, but reliability was night and day when we moved to fiber lasers. I can't comment specifically on the 920 systems since I'm not sure how they work internally (or if they even use the same mechanism for frequency shifting). I think the problem with going with 3 separate units is that it is going to get expensive. The Ytterbium models are cheap, but the frequency-doubled Erbium and the frequency-shifted 920 nm units are significantly more expensive, especially the Ti:Sapph class power. Not sure what Coherent charges these days, but you might end up spending more than it would cost for a tunable Ti:Sapph and a service contract that covers swapping out the Ti:Sapph when it gets into trouble. You may also have users complaining about not being able to hit lower wavelengths (for example, 740 nm for NADH), although 780 will work in many of these applications. Mike Giacomelli mgiacome@urrochester.edu

To echo Mike's comments, more lasers mean more expense and also more points of failure over their lifespan. A good single laser or integrated system with effective customer support from the manufacturer is the optimal condition. As an aside, I've watched the quality of some of the common brands of lasers slip over the last decade, and Mike's experiences mirror my own in several cases. Craig Brideau craig.brideau@gmail.com

One notable advantage of the proposed multi-line setup is that fast multi-color imaging (possibly line-by-line switching) is possible. The Chameleon requires time to tune to a new wavelength (seconds), so 99% of the time people use a single wavelength for an experiment. This may be different for lasers with dual output, but the "free" line (typically fixed at 1045 nm) is not the most useful wavelength. I haven't seen such a three-laser setup, but for a core facility it is important that the lasers are well integrated into a commercial microscopy system. Zdenek Svindrych zdedenn@gmail.com

If building a microscope for one very specific application that is known in advance, then fixed-line lasers are fine. But in a core, we never know what research needs will be required. We have different labs that work at 920, 930, and 940 nm. This may not sound like a big difference, but for continuity with past experiments or for second harmonics, being able to hit these subtle differences is important. Similarly, when using around 1150 nm, there are second harmonics that we can clip from the standard red filter set by tuning down or up a little. And we have customers, including chemists developing new probes, who want a wide variety of wavelengths for various reasons. I'm not an expert on the lasers but am expert on providing services across the spectrum. Which gets to a point about needing two wavelengths. If an investigator cannot wait 5 sec between fields while a laser retunes (and the software needs to be able to handle the pause), then two lasers are needed. Also, changing wavelengths may require refocusing collimators, other optical elements, or the objective itself to assure that the wavelengths are focused on the same plane in the sample. For live cell work, we like to have the lasers alternate for each line that is scanned (and some people might want the speed of simultaneous lasers, but then we get into a discussion of higher photon flux per time). We don't worry about the laser pulses being in synch, but for some applications this may also be important. Michael Cammer michael.cammer@med.nyu.edu

Non-Fluorescent Bright Field

Confocal Listserver

Risking a slight departure from the confocal microscopy topic; Do you know of any compounds that I may use as a non-fluorescent dye to increase the contrast in brightfield mode? In this case, specifically of PFA-fixed mouse brain slices? The goal is to make them more clearly visible on a brightfield system, while at the same time avoiding the introduction of fluorescence to keep the full spectrum available for other fluorescent markers. For example, hematoxylin/eosin and cresyl violet are common brightfield stains, but they introduce fluorescence. Thanks in advance! ***Jelle Postma j.postma@science.ru.nl***

Interesting question! I will point out that even if you find a dye that is not fluorescent, the fact that it works as a brightfield dye means that it is absorbing light. For example, a dye that appears blue is absorbing green and red light and transmitting blue light, which will still interfere with fluorescent measurements using green or red excitation. You may be better off looking at a transmitted-light contrast technique, such as oblique illumination, if phase or DIC are not available. On our stereomicroscopes, for example, we can adjust the angle of the incident light using a mirror below the sample, and with oblique illumination we get nice contrast for otherwise hard-to-see tissues or cells. I am not exactly sure this can be done on a standard brightfield microscope. Or perhaps closing the aperture diaphragm (so-called "dirty brightfield")? James Jonkman james.jonkman@uhnresearch.ca

DAB is not fluorescent, but it quenches fluorescence. So, it will mask any fluorescent dye you use to label the same areas. Sylvie le Guyader sylvie.le.guyader@ki.se

Many thanks, James and Sylvie. Good to know that DAB quenches fluorescence. Sudan Black staining seems to reduce background autofluorescence in some studies (sounds good). Brightfield images using that compound seem well contrasted! Provided there are no red flags for Sudan Black that I am missing, it looks like this might be useful. I will also check if we can use some of the suggested illumination modes. I love how it is called "dirty brightfield." Jelle Postma j.postma@science.ru.nl

Maybe try Trypan Blue? It should penetrate fixed tissue and make it darker. It may somewhat quench fluorescence because it has a broad absorption spectrum, but its concentration can be adjusted. Mike Model mmodel@kent.edu

As others have observed, this is an interesting question. The best bet may be phase-contrast or DIC as others have suggested, or simply to stop the brightfield condenser down while using a high-NA objective. Cheap, easy, and effective in many tissues. As others have noted, any dye will absorb light, potentially either in the excitation range or emission range of one or more of the fluorophores you want to see. Sudan Black will absorb fluorescence, which is why it is used for quenching lipofuscin autofluorescence. You might be able to get enough of a contrast-increase to make it worth it. So why do you not want to use a fluorescent dye? There are several DNA-binding dyes that span a large part of the spectrum. Do you really need all of the spectrum to be available for other labeling? Martin Wessendorf wesse001@umn.edu

Dear Martin and Mike, thanks for thinking along and for the practical tips! The requirement for the dye being non-fluorescent is indeed not set in stone. We could sacrifice a fluorescent channel for a tissue-level stain. To create a more standardized procedure for users here, and to keep maximum flexibility for them to use combinations of fluorescent stains, I am checking around to see if the idea of a nonfluorescent stain is even realistic. Equally for Trypan Blue and Sudan Black, it looks like they both absorb light, but if I go with this route to solve the problem, the hope is that enough of the target's fluorescence will still be able to see it. In short, the more fluorescent windows available, the more events of interest we can trace in the same brain slice. But the brightfield screening of slices before looking at fluorescence is performed on a simple system near the animals. Jelle Postma j.postma@science.ru.nl

A colleague who regularly pretreats brain slices with Sudan Black to suppress autofluorescence indicates it makes them a little black. Mike Model mmodel@kent.edu

A colleague of mine provided two interesting resources that are relevant to the question about brightfield stains: StainsFile, The Internet Resource For Histotechnologists by Bryan Llewellyn (https://stainsfile.info/xindex.html) and a book "Romeis - Mikroskopische Technik" that seems to be available only in German (https://link.springer.com/book/10.1007/978-3-642-55190-1). Andriy Chmyrov andriy.chmyrov@gmail.com

Eosin is fluorescent, but hematoxylin is not. So, hematoxylin-only staining might work. However, it does quench fluorescence as do other brightfield dyes. I think that more molecules of a brightfield dye are required to make it visible when compared to a fluorescent dye. Thus, quenching is probably unavoidable. There are several fluorescent channels available, that is, 1-DAPI, 2-green-GFP/488, 3-orange-Cy3, 4-near red, Cy3.5 or AF594, 5-far red - Cy5 or AS 635P, 6-far far red, AF 680 or CF680R, without considering spectral or lifetime unmixing. So, DAPI plus 5 different antibody stains are possible. Some investigators want several fluorochromes, but when considering the number of structures in a sample that can be stained the numbers often crumble. There are a limited number of hosts for primary antibodies. Steffen Dietzel lists@sdietzel.de

Colocalization Maximum Intensity Projections (MIPS)

Confocal Listserver

Hi everyone, I'm looking for good analogies, tutorials, lessons, graphics, ideas, etc., to convince our users that they should be using our Imaris license to determine colocalization in confocal Z-stacks instead of maximum intensity projections (MIPS) in FIJI. Since they see 'differences' in treated versus untreated in their MIP colocalization analyses, they are happy. I'm pulling my hair out that their analyses are not done properly, and we have software that can handle 3D data sets. Are there any good resources, publications, or data sets to share to show why investigators should NOT be using MIPs to quantify colocalization in confocal Z-stacks? My hand-waving representations and hand-drawn artistic abilities don't seem to be convincing enough. Thanks so much. ***Kathryn Spencer kspencer@scripps.edu***

An orthogonal section (XZ, YZ) of their 3D stack should show that many 'colocalizations' in MIPS are actually overlays of foci that could be microns apart. This manages to convince most of our users. Fred Indig indigfr@grc.nia.nih.gov

The method used to most efficiently answer colocalization depends on the question being asked. For example, if only curious about lateral colocalization in two dimensions, then a MIP is valid. For 3D non-parametric colocalization (the more common type of colocalization many are interested in), one of the easiest ways is to binarize the two channels of interest, make an AND stack of the two stacks, and then measure the ratio of the AND stack mean intensity to the mean intensity of the channel of interest. 1 = perfect colocalization, 0 = no colocalization. Benjamin Smith benjamin.smith@berkeley.edu

Please look at the top section of http://microscopynotes.com/imagej/colocalizationspotssimulation/index.html for an il-

lustration of why MIPs are dangerous. The percent overlap based on a simple threshold followed by simple AND math is very easy to achieve in ImageJ and other software. Including removing features such as stained nuclei from the analysis. Michael Cammer michael.cammer@med.nyu.edu

A MIP is a worst-case scenario for quantitation since it deletes data, and it removes a dimension that may have useful information in it. As long as background subtraction and noise correction (that is, with deconvolution) are properly performed, an average or sum projection will at least do the job non-destructively. Honestly, if people are going to take a Z-stack and collapse it, then they might as well do the same thing in one shot on a wide-field with a lower NA objective if they can get enough lateral resolution. Adding a dimension can add power, but it adds complexity and makes segmentation more challenging. It's not bad to start with a 2D plan and add the third dimension when the experiment needs it. On the other hand, you can't just use the heuristic of asking whether 2D analysis returns a clear difference between control and experimental data! That skips the important step where experimental artifacts created by the projection process are determined. Take, for example, a treatment that makes epithelial cells taller or denser, that is, by rounding up. Taller cells mean more information gets compressed or lost in a Z-projection. Taller cells compressed into a MIP will have a higher average Pearson's or Mander's score, even if the 'real' proximity of proteins was not changed at all. Doing a 2D analysis without considering artifacts can lead to embarrassing reversals that most investigators want to avoid. Before going to 3D, I'd also ask whether a system has enough Z resolution to make a useful distinction! If you picture the 'fried egg' profile of a cell on a flat coverslip, even a confocal with a 1.4 NA objective has a hard time telling the upper and lower cell membranes apart from the cytoplasm in between them (aside from very close to the nucleus). Super resolution techniques (or TIRF) can do better, but it's important to have a rationale for when to put in the extra time for 3D, or add even more extra time (and data volume!) and step up to super res. Timothy Feinstein tim.feinstein@gmail.com

For fun, I went ahead and made an ImageJ macro that can demonstrate the difference between 2D and 3D colocalization, as well as the impact of object size on the degree of colocalization: https://www.youtube.com/watch?v=DjjL2EZxWCk. The raw video can be found here: https://bit.ly/3uABSd7. The macro used to create the movie can be found here: https://github.com/Llamero/Colocalization_demo_macro. In the macro, the size of the spheres and the degree of overlap can be adjusted to demonstrate different concepts and issues. The macro requires the 3D library and TransformJ library to run. Ben Smith benjamin.smith@berkeley.edu

This is a fantastically simple and effective demonstration. I appreciate the examples. These are good analogies to help convince the lab that using MIPs is completely inappropriate for their experiments and what they want to quantify (pre-synaptic and post-synaptic colocalization in 3D tissue). Not to mention the fact that they have the confocal pinhole open to 2X Airy and step size at 3x Nyquist. And yes, we are scheduling a lab-wide image analysis tutorial consultation soon. On a side note, a post-doc showed me the published reference where he obtained these specific imaging parameters and colocalization quantitation workflow. So, I'm fighting against an already published paper. Kathryn Spencer kspencer@scripps.edu

Quantifying colocalization is a minefield. MIPs are clearly nonsense and will change as the thickness of the Z-series increases. 1) There are too many colocalization coefficients and some are almost meaningless. In a recent article we made a case for abandoning one group of coefficients. The premise is that colocalization coefficients fall into two useful groups, those that measure co-occurrence, the degree to which molecules are found in the same place, and correlation, the intensity relationship when fluorophores found together. Both measures are informative. However, this scheme exposes a third group that combines the two types of measurements into an unintelligible mess that we propose dumping as the measurement can arise from widely differing distributions (https://doi.org/10.1002/cyto.a.24336). 2) A second serious problem of noise arises with point scanning images as two images taken consecutively of the same fluorophore are not measured as being perfectly correlated. So, if two nominally identical images don't correlate perfectly then correlations from two different fluorophores will not be measured correctly. We demonstrate a practicable solution in https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2818.2008.01967.x. 3) Expressed proteins with a fluorophore can cause problems. Colocalization can be measured accurately, but the levels of expressed protein are usually superimposed on the endogenous protein and will distribute differently. Binding sites are saturable. Overall, there are serious problems to resolve including segmentation. Jeremy Adler jeremy.adler@igp.uu.se

I would support the fact that colocalization of MIPs is not appropriate. I would add that before doing colocalization studies, deconvolution should be applied to the volume of interest. Louis Villeneuve louis.villeneuve@icm-mhi.org

Everything shared by Jeremy provides a great overview of the problem. Just to add, there are a bunch of resources on the Imaris website which try to make this topic as easy as possible to follow. Most recently, our American (East) support team member Matthew Gastinger did a great webinar on how to use Imaris to look at colocation and colocalization (both voxel and object based). You can find that here https://imaris.oxinst.com/learning/view/article/various-ways-of-solving-the-colocalization-problem-in-image-analysis. It also sounds as if you need an example to get the message across to users that may need help understanding the fundamentals. If they are having a tough time understanding, I would use a picture taken from an Ames Room Model. While not particularly scientific in the biological sense, it is a fairly useful tool to explain how you can't trust perspective in 2D. I previously used it in presentations when I was a post doc to explain why 3D image analysis was important. Nick Jones n.jones@bitplane.com

My go-to illustration of the unsuitability of MIPs, and the effect of cell geometry and anisotropic voxels in colocalization analysis, is figure 2 in this article from 2008: https://pubmed.ncbi.nlm.nih.gov/18353895/. Chris Wood chris@ibt.unam.mx

As the discussion is spinning a bit off topic, let me contribute. There are far worse things than MIPs. I often encounter colocalization within the nucleus (transcription, translation, repair, etc.), see, for example, https://drive.google.com/file/d/1n91JKGrhVgN3YHW9luAbVUpAHINNRljg. While Pearson coefficients are one of my favorites (insensitive to gain, offset; no need for thresholding), the dark pixels do indeed count! What's shown in the snapshot cited above is while the two labels (green and red) hardly colocalize in the nucleus (Pearson=0.19), when other

structures are included, such as nucleoli (Pearson=0.67), or even the outline of the whole nucleus (Pearson=0.82), the numbers are clearly way off. This can be (sort of) avoided with the threshold-based methods if one is meticulous and makes sure only the spots are thresholded and not the entire nucleus. Tricky! For what it's worth, the numbers above were generated with this simple ImageJ macro that just calculates the Pearson coefficient between the first two channels in an image (within a rectangular selection of choice): https://drive.google.com/file/d/1Ft1CS5uFElakRft-W3gJLSrgVbf5WHhm. It is a viable high-throughput alternative to the overly complicated colocalization plugins in Fiji. Remember, the numbers obtained have absolutely no meaning unless compared to appropriate controls, more controls, and still more controls! Zdenek Svindrych zdedenn@gmail.com

Hi Zdenek, I have to voice my strong disagreement with part of your statement "While Pearson coefficient is one of my favorites (insensitive to gain, offset; no need for thresholding), the dark pixels do indeed count!" For offset and gain you are correct. Within a wide range they are unimportant. However, the assertion that there is no need for thresholding is wrong. You are correct in stating that they do affect the measured correlation. 1) Consider a negatively correlated dataset, where one molecule is abundant, and the other is weak - and vice versa - on a scatterplot with a nice -45-degree line and an appropriately measured negative Pearson correlation. Now include some pixels with neither molecule. These form a nice cluster at the bottom left of the scatter plot that is disconnected from the -45-degree set of points. The Pearson correlation now becomes much more positive with the shift depending on how many empty pixels that are included. The number of empty pixels affects the measured correlation despite there being a negative relationship wherever both molecules are found together. 2) Conceptually, pixels with neither molecule provide no indication about the relationship between them when both are present. This is usually what we are really interested in. The empty pixels simply report that there are no molecules in an area, which is adequately reported by coefficients that measure co-occurrence. Area of overlap, M1 and M2, including empty pixels, increases the measured correlation except when the correlation in the population of pixels is near perfect. Then it has no effect. 3) Try this. A simulation where two images each have blobs with random intensity thrown in at random positions and Pearson is repeatedly measured as the images fill with blobs. The algorithm means that *a priori* we know there is no correlation between the intensities in the two images, which is exactly that we find if the Pearson Correlation is measured only from pixels in which both molecules are present. If empty pixels, or pixels with one of the two molecules present are included, a quite different Pearson Correlation results. 4) Or this: two molecules are uncorrelated with high intensity resulting in a nice cloud of points in the center of the scatterplot and the measured correlation is around zero. Add a cluster of empty pixels and the combined correlation becomes strongly positive, even though each population measured alone is around zero. 5) The problem with empty pixels is the values for both molecules are similar even though neither is present. Worse, the calculation of the Pearson Correlation involves subtracting the mean intensity over the ROI from the values, so the empty pixels make a large contribution to the measured correlation. In the first two scatterplots you showed the correlation is clearly positive, but the empty voxels increase the magnitude of the correlation. This is discussed at length in http://doi.org/10.1371/journal.pone.0111983. Jeremy Adler jeremy.adler@igp.uu.se

I just skimmed this paper and it seems very readable for students, etc. However, I think that a key sentence, "Voxels larger than those specified by the Nyquist criteria will under sample the image, create artifacts, and result in false colocalizations," requires clarification. I would not say FALSE colocalizations; I would say colocalizations limited by the sampling rate. The question is to what extent the colocalization is biologically relevant and this varies based on the questions being asked. For instance, a few weeks ago a student came to me with images of puncta in cells that were clearly colocalized. This was accurate for the images she had that were taken with a low N.A. 20X lens. Within the limits of the imaging modality, the molecules of interest were colocalized and this may have been a meaningful answer. It definitely was a meaningful answer at the sampling rate of the system, and it provided valuable information about localization at the organelle scale within individual cells. But it was not the answer she needed because her biological question was one that can only be answered by a higher spatial resolution. Switching to a 63X N.A. 1.4 lens clearly showed that the green and red were mostly in different structures. Maybe they were always in different structures or possibly the same structures sometimes? What's next, FRET, SMLM? And, of course, other types of assays such as co-IPs. Michael Cammer michael.cammer@med.nyu

You seem to have already enough material to address the problem of using MIPs and to measure colocalization. I personally like the YouTube movie of Ben, and the suggestions to include the correction of noise and blur (deconvolution). However, these imaging artifacts are not necessarily the main contributor of false colocalization results. Channel shifts and crosstalk should definitely be considered when measuring colocalization (pixel or object based). Hot pixels/cold pixels, drift during acquisition, chromatic aberration, and undersampling are also spoiling this type of analysis. With 100 nm TetraSpeck beads imaged with a confocal (36 x 36 x100 nm sampling), all 4 channels should overlap and give a Pearson value that is close to '1'. However, we measure values far from that (even below <0.5). See the example image at the bottom of this webpage: https://svi.nl/ColocalizationBasics. There are also animations on this same page and https://svi.nl/BlurAndNoiseAffectColocalization that explain other imaging pitfalls as well. Vincent Schoonderwoert vincent@svl.nl

Gold Palladium Target Composition

Microscopy Listserver

Dear all, what are the relative amounts of gold and palladium in a Au/Pd target? I used one for the first time yesterday and was surprised to see a peak for Au, but not Pd in an EDS analysis. Thank you in advance. ***Stephane Nizet nizets2@yahoo.com***

In most cases the Au/PD ratio is 60/40. What kind of EDS analysis did you do? Point scan? Maybe try an area scan. Rohan Prakash rohan.prakash14@gmail.com

It usually has 60% Au and 40% Pd. What else was in the sample? If there is another peak at around 21keV and 3keV, you would not be able to see a Pd peak. Sayit Uğurlu sayitugurlu@gmail.com

This is a good example of when to use DTSA-II or Electron Flight Simulator. Also, you want confirmation the 80/20 or 60/40 Au/Pd target is weight% and not atomic%. You did not mention your electron beam energy (KeV), substrate, or expected coating thickness. Jim Quinn james.quinn@stonybrook.edu

Au/Pd targets are usually 60:40 or 80:20. This raises a few questions that are beyond my knowledge: How does the proportion of Au and Pd in the target alloy affect the efficiency of sputtering and the grain size of the coating? Is there a "eutectic" alloy composition for sputtering that is most efficient? Does the presence of Pd inhibit crystallization of Au in the coating (and so result in a finer grain size)? Kurt Friehauf friehauf@kutztown.edu

Quorumtech provides Au-Pd targets at a ratio of 80/20: https://www.quorumtech.com/wp-content/uploads/2021/01/Q-Plus-Series-options-and-configs-V1-1.pdf. You may also find 60/40 Au/Pd targets at other manufacturers. The elemental composition/catalog number of a target is typically specified on its packaging. Inna Popov innap@savion.huji.ac.il

Cross-Sectional Electropolish

Microscopy Listserver

Can anyone recommend good references (literature or web) for cross-sectional electropolish protocols for metals? I am imaging dice, sandwich, dimple, and then twin-jet samples looking at dislocations in ion-beam-implanted metallic samples. The FIB leaves far too much dislocation debris behind to get the data I need. Thanks. ***Chad Parish parishcm@ornl.gov***

The main issue may be bonding in the cross-section. Most bonding agents (M-Bond 610, Gatan G-1, Epotek 353ND) are insulators and won't e-polish. It is possible that a thin enough glue layer may dimple sufficiently, and the glue layer won't be an issue. I suspect that you will not get much thinning immediately adjacent to the glue layer, but you should be okay a little further away. My suspicion is that mixing metal powder into the glue layer will not help. While the glue will then be macroscopically conductive, there will still be small non-conductive regions. Also, the glue layer will likely be thicker. Henk Colijn colijn.1@osu.edu

When I worked at Monash/MCEM years ago, Xi-Ya Fang was a pro with electropolishing. I would encourage you to reach out to her: https://www.monash.edu/researchinfrastructure/mcem/about-us/people/dr-xi-ya-fang. Ellen Lavoie lavoie@uw.edu

SEM Imaging of Fe Nanoparticles

Microscopy Listserver

I am trying to determine the average particle size from a conductive film with embedded iron nanoparticles. It is very hard to get a clear image as I increase magnification. Could the nanoparticles be magnetizing and blurring the image? TIA for any advice. I don't get many materials science samples in our facility. Best, ***Julian Smith III smithj@winthrop.edu***

First, are the Fe nanoparticles embedded in the top of the film, so they are sticking up out of it, or within the film and covered by it? If the latter, that will make the boundaries indistinct. Second, from your post, I infer you are using secondary imaging. True? Or backscattered electron imaging (BSE)? If you are not using BSE, try that. The Z-difference between the film and the Fe particles will help clear up the edges. I would also raise the stage as close to the BSE detector as you can. Assuming it is an under-lens detector, and not off to the side. Plus, try different spot sizes and accelerating voltages. 20kV should be good, but try 25kV? Note: Try sputter-coating both surfaces of the glass substrate so that the paste is applied to a conductive surface. This will provide a better path to ground for the electrons and better imaging than just connecting the top surface of the film to ground. Also, I have found conductivity problems (therefore charging) with TiO, and I doubt adding Fe will help all that much. Is this substrate required? And what is the paste? What is its conductivity? Just to ensure your life is difficult enough, how are you doing stigmation? If stigmation correction is close but not correct, then the image will look fuzzy, but not smeared, as it is when the stigmation is obviously off. Given that beam spread is affected by depth into a sample, you can have an interesting time getting stigmation correct on an imaging volume at some depth into the film, even if it is correct at the top surface of the film. Just to be more annoying, how accurate do the particle sizes have to be? Non-fuzzy images or not, you have a "where's the edge" problem, and therefore a real measurement accuracy problem. Not to mention trying to get the interaction volume correct. Which is a reason to ask, are you sure the SEM is the correct instrument for this? Do you have a confocal, maybe with STED or other super-resolution feature? This could be more accurate. Phil Oshel oshel1pe@cmich.edu

Thanks for the responses! SEM, W gun, 20kv. Spot-size set to 35 and 10mm WD, which is what we typically use for high-res work and EDS maps. Column aligned, focus and stigmation set properly. Particles were applied in a paste to a fluorine-doped titanium oxide (FTO) substrate on glass, and then the substrate and film were baked overnight at 550°C and grounded (film surface to stub) on aluminum stubs with silver paint. I still get a carbon signal in the X-ray spectrum, so maybe the problem is that it needs to bake out longer. But I cannot get sharp images of the iron nanoparticles. They are quite fuzzy at the edges. Hence, I am wondering about magnetic field effects. Julian Smith III smithj@winthrop.edu

20kV acceleration voltage and using the SE detector might bring too much signal from within the nanoparticles, hence the fuzzy edges. Also, if your specimen is mounted on a large piece of glass substrate this might add some strange effects, even when silver paint is applied on the edges. I would try to do this task with a field emission SEM and 2–5 kV. Or on your SEM with as low a kB as possible using a beam diameter as narrow as possible. Stefan Diller diller@stefan-diller.com

If the paste is carbon-based, you should be able to see the Fe contrast using a backscatter electron detector. You may have to reduce the spot size considerably and take a prolonged exposure, but there should be good contrast. It may still give you a noisy image, but you should see the particles clearly. Carol Heckman heckman@bgsu.edu

I agree with Stefan, try reducing the keV for imaging the nanoparticles. I would also using a short working distance, something like 4 or 5 mm. I think that magnetic effects from the nanoparticles would manifest similarly to charging (streaking in the image) as the beam moves while scanning. Brittany Cymes bacymes@gmail.com

Lowering the kV will work well if the particles are on or very close to the film surface. Otherwise, you'll just be imaging the film. If you have Casino or DTSA-II, you might model the beam penetration into the film and see what kVs best reach the particles, and how much the electrons scatter within the sample. Phil Oshel oshel1pe@cmich.edu

I would like to second Phil Oshel's comments. Voltage and signal are both critical. I have some images collected from particles dispersed in a polymer matrix (ftp://ftp.marl.iastate.edu/_Gallery/Voltage%20effect/). The particles are generally below the surface. Substantial voltage is needed to get a good outline of the particles. There is a big difference between 20 and 30 kV for the GaIn particles. The BSE signal provides more particle info than does SE. (Refer to the TiO_2 samples at 10 or 20 kV.) A simulation of the different signals at different voltages would be helpful, especially if you can model the particle in its environment. It would be good to have a dedicated BSE detector rather than using the BSE mode of an ETD secondary electron detector. The latter is a poor substitute. For a pole piece-mounted detector, shorter working distances are good. We normally image samples at 10 mm working distances in our Quanta. I will shorten the distance for samples with weak signals. However, I am careful not to shorten it too much or I lose signal strength. More of the BSEs head up the column than hit the detector. Of course, for in-column detectors, short working distances are good. We don't know much about particle size or loading. We were told that " Particles were applied in a paste to <snip> FTO substrate on glass". For the record, FTO is fluorine-doped tin oxide, NOT titanium oxide. It is used to provide conductivity to glass substrates. It is also not particularly smooth. It clearly has texture at high magnification. It is good that the film was grounded. FTO renders the glass surface conductive. It does not render the thickness conductive. You need a bridge across the glass thickness. It would be nice to know what size the particles are and what liquid was used to make the paste. You say the film was baked overnight. It seems that you do not want to have any carrier left afterwards. You could have a double-bind there. If the carrier is fully volatile, heat alone should remove it with temperature. However, if it needs oxygen to burn the carrier off, then you also run a risk of oxidizing the metal particles. You may be trying to find a temperature that effectively completes the one without starting the other. There is likely no sharp temperature cutoff. Even if the carrier is volatile, it probably has components that will remain at high temperatures. Have you tried cooking the carrier alone on a clean substrate in the absence of iron particles? Do you truly end up residue-free? I predict not. That is probably the reason for C in your X-ray spectrum. So, I am guessing that you are looking for a layer of only Fe nanoparticles. The carrier should be all removed. However, I suspect you have a residual organic meniscus around the Fe particles. You may not be able to distinguish the difference between Fe and organic material in SE. You should see a clear difference in BSE at the right voltage. Too much voltage (like 20 kV) and you will blast through the meniscus and just see fuzzy edges. Too low a voltage (perhaps 2 kV) and you will image only the surface of the meniscus. We commonly start at 10 kV and work from there. Multiple voltages may be beneficial. Unless your particles have their fields aligned with each other, I really don't expect magnetism to be a problem. We have looked at magnetic samples successfully. They need to be kept small or I run out of stigmator correction, and that is clearly obvious. Warren Straszheim wesaia@iastate.edu

Sending samples through the mail

Microscopy Listserver

Hello all. Has anyone had experience receiving or sending EM samples through the mail? We have users that want to send us tissue in fixative, but I am not sure what to tell them. Any suggestions are welcome. Thank you. ***Rebecca Jackson rebecca.jackson@utsouthwestern.edu***

We have. We instruct our clients to fix the samples as usual, do the buffer washes, and ship the samples to us in the buffer. Leona Cohen-Gould lcgould@med.cornell.edu

I do not know if there are regulations in your country that prohibit sending specimens in aldehydes through regular mail. If so, shipment of aldehyde-fixed specimens in buffer may be a solution. I recommend including a sodium borohydride treatment step in the protocol to avoid possible negative effects on ultrastructure. When using formaldehyde or a mixed fixative with formaldehyde and a low concentration of glutaraldehyde, remember that fixation may be reversed over time if the specimens are stored in buffer. This can be prevented by including a 0.1–1% sodium borohydride step in the protocol, see, for example, https://journals.sagepub.com/doi/pdf/10.1177/31.2.6339606. Peter van de Plas p.vandeplas@aurion.nl

We work with samples sent through the mail all the time (endometrial tissue for SEM analysis). We provide the doctor a vial containing the fixative (we use 2% glutaraldehyde in PBS). The doctor places the tissue in the vial and sends it to our lab. We analyze a portion, and the rest of the tissue is stored in the same fixative. We have analyzed tissue stored for >10 years in the fixative and it was intact. Yorgos Nikas eikonika@otenet

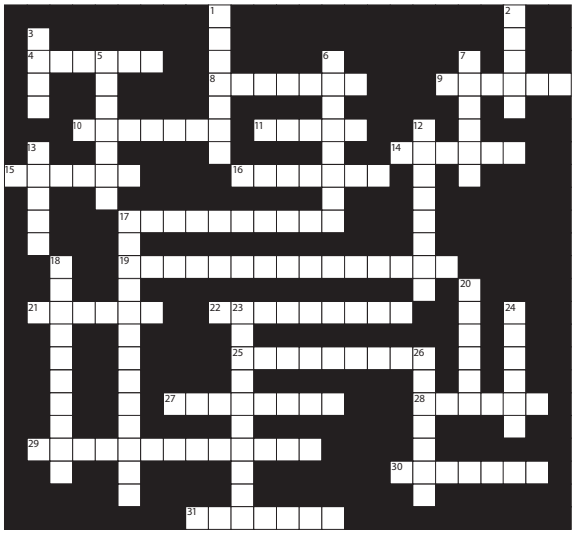
Thank you for the information! I will look into adding sodium borohydride if we need to ship in buffer. Rebecca Jackson rebecca.jackson@utsouthwestern.edu

MT

Crossword Puzzle Answers

Surprise! This issue's crossword puzzle is a contest for attendees of Microscopy & Microanalysis 2022!

See page 55 for contest rules.



Answers to the July 2022 crossword puzzle will appear in the September 2022 issue of *Microscopy Today*.

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