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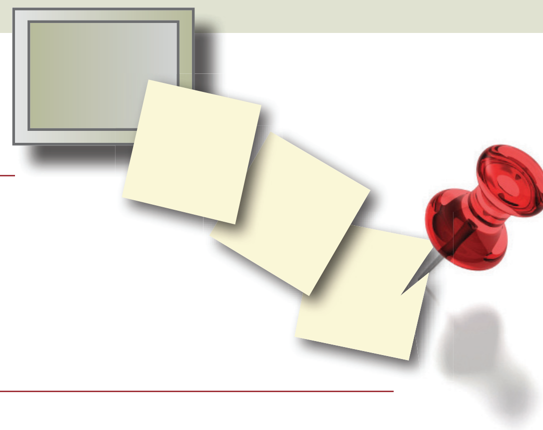

NetNotes

Edited by Bob Price
University of South Carolina School of Medicine
Bob.Price@uscmed.sc.edu

Release of Bio-Formats 6.8.0

Confocal Listserver

Dear all, we have released Bio-Formats 6.8.0, which includes new packages in addition to several updates and improvements. Full details can be found at https://docs.openmicroscopy.org/bio-formats/6.8.0/about/whats-new.html and the software is available at: https://www.openmicroscopy.org/bio-formats/downloads/. In addition, it will soon be available from the Java-8 update site for Fiji users. If there are any problems or comments, please use the Image.sc forum (https://forum.image.sc/tags/bio-formats). Regards, ***The OME Team d.gault@dundee.ac.uk***

Multiscale Sample

Microscopy Listserver

I've been charged with finding one, unifying sample, that would be interesting to grade-school age students and be compatible with SEM, AFM and TEM. The plan is to produce a video in place of our usual in-person outreach activities. The SEM and AFM seem straightforward but finding meaningful data from the same sample with the TEM is proving difficult. The imaging tools I have available are Bruker Dimension Icon AFM, Zeiss Merlin SEM, Helios G3X FIB/SEM with Quorum Cryo attachment, and Tecnai Osiris 200 kV TEM/STEM. Any suggestions would be helpful! ***James R. McBride james.r.mcbride@vanderbilt.edu***

Bacteria like *Bacillus*, *Pseudomonas*, etc., and especially ones with lots of flagella. They are a good size (~1 µm) with interesting information related to health, gut flora, and environmental importance. They also work at all scales, even light microscopy, so the grade-school kids can look at them with the school microscopes. The AFM would be good to use on the flagella. Philip Oshel oshel1pe@mich.edu

If you don't want to use biological samples, you could use Au nanoparticles approximately 50 nm in diameter. These are easy to image with a TEM. For an SEM, use a holder that accommodates grids and probably use BSE mode. Not sure how an AFM will work and that may determine the optimum diameter of the particles. Richard Hailstone hailstone@cis.rit.edu

Any particulate material should work. Bacteria are a great idea but mineral powder, for example, would be much easier to prepare and very exciting to observe too. Observe the differences in shape between salt crystals, dirt particles, clay, quartz and so on. Careful with powders made of magnetic material, though! Have fun! Stephane Nizet nizets2@yahoo.com

You might consider (acid-cleaned) diatom valves. They have a wealth of detail at a large range of magnifications. Very commonly viewed with TEM and SEM, but I've also seen AFM images. James Ehrman jehrman@mta.ca

Since you list a chemistry department, then I would stick with "materials" samples. You will have a better chance of correctly describing them. Gold crystals have always been the easiest demonstration sample for me as they require the least sample prep. Jim Quinn jquinn11733@gmail.com

Bacteria and diatoms are some great samples, but I also have used butterfly wings to demonstrate scaling ranges between microscopes. All kids can relate to them, and they are cool samples to image. Sophia Hohlbauch sophia.hohlbauch@oxinst.com

Spatial Phenotyping

Confocal Listserver

I am interested in finding a better (easier) way to immunophenotype and perform single-cell spatial analyses in frozen and FFPE samples. We've been using cyclicIF and this works but is not necessarily the best option for an imaging core. A barcoded system seems to be the way to go. I have been looking into the CODEX system, but know this group is great to ask for insight, suggestions, and a larger perspective regarding spatial (immuno)phenotyping and analysis options. ***Heather Jensen Smith heather.jensensmith@unmc.edu***

There was a recent publication that covers a variety of multiplex imaging systems and why you might choose one over the other: https://t.co/Ra0fAoIPmP. I have also created a Twitter thread summarizing a bit of it here: https://twitter.com/IAMichaelNelson/status/1465728407610798090?s=20 which also includes a link to the non-paywalled preprint version at the end. Mike Nelson msnelson@gmail.com

Thanks Mike, for sending this. Very helpful overview and key perspectives in this Nature Methods article. Here is some additional information for others that may encounter issues: JW Hickey et al., https://doi.org/10.1038/s41592-021-01316-y. Epub ahead of print. PMID: 34811556. Heather Jensen Smith heather.jensensmith@unmc.edu

We've had MERFISH running in my facility with the Vizgen platform for the past 7 months. We're also considering CODEX or similar technology to extend our antibody multiplexing capability beyond our current 8-plex. Christina Baer christina.baer@umassmed.edu

Calibration of Pixel Size

3D Listserver

*Dear colleagues, I would like to check/correct the pixel size on our TEMs and am open for suggestions to make my life as easy as possible. For low mags I have the venerable cross grating with a spacing of 463nm. My plan is to take images and use the cross-correlation function from Digital Micrograph (DM) to get a precise spacing in pixels. For high and ultra-high mags, I have the MAG*I*CAL that offers calibrated distances in the range of 10nm/100nm/1000nm/4µm. My plan is to use Fiji or DM to make manual measurements. Does anybody*

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have better suggestions, procedures or calibration standards that work well? Thanks in advance. ***Tobias Furstenhaupt furstenh@mpi-cbg.de***

I tend to use Mag-I-Cal Si reflections. Especially with our K2/K3 systems using super-resolution, one can cover quite a large range of magnifications. I manually measure line profiles in FFTs. For lower mags the cross grating will do, and if using SerialEM, one can automatically acquire montages of cross gratings at higher mags by binning (that is, bin 4). This gives small enough image montages. Another option is thallous chloride (commercially available) as its spacings allow calibration of lower magnifications. Be aware of linear distortions when calibrating, but on the Titan these should have been corrected. On other microscopes this is not possible, and measurements can be off up to a few percent depending on the direction measured in Fourier space. I have tried Dave Mitchell's free DifTools plugin for Digital Micrograph for the Mag-I-Cal images. It may find peaks in FFT. Be aware that calibrating using the Cross Gratings 463 nm repeat can be off a few percent. In the end, for the typically used higher mags, I prefer a few decent images from an apoferritin grid and letting Relion or CRYOsparc give an accurate number assuming the vendor specified Cs. Wim Hagen hagen@embl.de

We recently wrote an ImageJ macro for performing magnification calibrations using cross grating grids (using the 463 nm spacing at low mags and the gold ring in the FFT at high mags). The macro is available at https://github.com/directelectron/imagej-macros/blob/main/DE_DQE0.ijm. Benjamin Bammes bbammes@gmail.com

I want to add some details about "calibration samples". Generally, there are 3 types of samples for calibration. (1) Calibration check samples, (2) Reference material samples, and (3) Certified reference material samples. Type 1 samples include those made for verifying if previous calibrations are correct. These samples do not offer any fundamental physical parameter and are made to represent a "generally trustable dimension". They have no accuracy/precision guarantee or traceability to any standards institution (NIST, BAM). For TEM magnification calibration, cross grating and shadowed latex samples can be placed in this category. The mold for making cross grating replicas is made using a known laser wavelength but the stability and changes in the replica film during preparation is not verified by a standards institute. We just trust them. Type 2 samples include those with a defined physical or natural dimension which are globally constant, such as the d-spacing of pure crystals or certain laser wavelengths. These have been measured many times by reputable organizations. For TEM calibration, pure compounds such as Au, Pt, and Pd, and compounds such as thallium chloride and refractory metal carbides, are in this category. When searching for the parameters of such materials, some considerations must be accounted for, such as sample temperature during calibration and vacuum conditions. Note that non-stoichiometric but pure alloys and compounds such as Pd-Pt, Pt-Ir, Si-Ge and evaporated silicon oxides are not reference materials and should not be used for this purpose. Here is a list of commercially available recommended reference materials for TEM calibration:

1-Gold on Carbon: Ted Pella 613 or Agar AGS132

2-Oriented Gold film: Ted Pella 646 or Agar AGS135

3-Thalium Chloride: Agar AGS110

4-Aluminum: Ted Pella 619 or Agar AGS108

Should you want to make a sample, any refractory, chemically stable, non-magnetic, and pure compound is preferred. For example, platinum group members are ideal for this purpose. I am not aware if commercially available samples such as asbestos (Crocidolite), copper phthalocyanine, graphitized carbon, or protein particles (for example, ferritin or catalase 2D crystals) might also be considered as a reference material as they are not rigid crystals with defined and constant d-spacings. Also, some samples like potassium chloroplatinate have specific directionality which require high tilt to reveal the desired lattice fringes and are not advisable. One should avoid a high defocus and provide a parallel beam (as much as possible) while calibrating using lattice fringes. Type 3 samples are not fundamentally constant but have been examined by a standard center and compared to another known sample (either another reference or another certified reference material). This method of hierarchical credibility and validation is named traceability. For TEM, we have multilayer X-ray monochromators (normally made using MBE or similar coating methods) or similar structures made for this purpose such as BAM-L002/XXX or Norrox Scientific Mag*I*Cal. BAM-L002-XXX is in bulk form and needs to be prepared using a FIB-SEM. Also, there are some traceable and calibrated beads (latex, glass, silica, etc.) which, despite their traceability and high accuracy, do not offer high precision (for better understanding of accuracy versus precision, please see: Accuracy and precision - Wikipedia). If using a scope for high resolution cryoEM single particle analysis, super accurate or traceable calibration is not needed as there are many structures in a sample that can be used to correct the model after image processing, refinement, and subsequent model building. Counterintuitively, good calibration of a TEM at low mag is more difficult than high mag. At higher mag, image distortion is normally negligible and there are many reference materials. At low mag, most machines suffer from image distortion (especially those with energy filters) and suitable reference materials become scarce. Also, if using lens-coupled cameras or fiber optic-coupled CCD (or CMOS) the image should be examined for possible optical distortion. Farzad Hamdi farzaad@gmail.com

Calibration Slide for Focus Height

Confocal Listserver

Dear all, does anybody know of a commercially available calibration slide for measuring focus height that can be used in bright field microscopy? Alternatively, how do you quantitatively calibrate the focus height in bright field microscopy? ***Shigeo Watanabe shigeo-w@sys.hpk.co.jp***

Perhaps there are other solutions but look at the PSFCheck slide. It has a nice 3D array. https://www.psfcheck.com/psfcheck-slides Mika Ruonala mika@icit.bio

We sometimes put a mark on the top and bottom glasses separated by a distance and fill the gap with a solution of an absorbing dye. We then compare the focal positions as read by the microscope (adjusted for refractive shortening or elongation), with the depth calculated from absorbance. I can send you some papers if interested. Mike Model mmodel@kent.edu

You should look at Working Group 6 of the Quarep initiative for microscopy quality assurance: https://quarep.org/working-groups/wg-6-stage-and-focus-precision-and-other/. The Quarep group (I am a member of the power stability group, WG1) has the overall goal of developing calibration and quality assurance techniques for microscopy. WG6 is working on sample positioning and calibration issues, so they would be the experts for best practices on this topic. Craig Brideau craig.brideau@gmail.com

I used a similar approach: with a long working distance objective (say, 10x dry) I mark the top and bottom of the glass slide with a marker and measure the thickness of the slide with a machinist's

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micrometer. Focus on the top and bottom mark, noting the focus positions in the software and calculate the difference, let us call it "Dz_motor". For a dry lens looking through 1000 micrometers of the soda lime glass slide (refractive index 1.523 at 589 nm, as listed for ThorLabs slides), you can check whether the difference between focus positions in the microscope software is the same as expected from this calculation: Dz_motor=Dz_sample*n1/n2 where Dz sample is the slide thickness measured by the micrometer gauge, n1 is the refractive index of the immersion media (air, ~1.0) and n2 is the refractive index of the glass slide (1.523). This only gives you the average number and will not tell you the error in the individual steps of the focus drive. Here is my Hillbilly Engineering approach to measuring individual steps of the z-motor with reflected light illumination. With a Mirau interferometric objective, image a microscope slide with a cover glass on top that has one end sitting on another cover glass of known thickness. Then calculate the height difference of the wedge surface for the field of view in the camera. The Mirau objective will produce a series of fringes that are perpendicular to the slope of the wedge. When changing focus, particular dark fringes will move by a certain distance along the slope since a different area of the wedge now has the same exact distance from the objective. There are probably better ways to do it. Stan Vitha vitha@tamu.edu

Not quite sure what you are trying to do but a dial indicator is accurate to about 2 microns (or maybe better). Mark Cannell mark.cannell@bristol.ac.uk

We use 3M double-sided tape of known thickness as spacers between 2 coverslips: 9415 (80µm) and 467MP (50µm) and match the refraction indices of the objective immersion medium and the sample. Sylvie le Guyader sylvie.le.guyader@ki.se

DMP Rapid Dehydration

Microscopy Listserver

I would like to hear from anyone who is currently using DMP rapid dehydration protocols. In my literature search I have found papers (including the first one by Muller and Jacks, 1975) mainly from the 1970s and 1980s with a couple from the early 1990s. Is this technique still commonly used? It is not commonly found in the standard reference works on EM. ***Tom Bargar tbargar@unmc.edu***

I use it when working with very difficult to dehydrate samples. When I was a grad student working with Wayne Fagerburg at UNH we discovered (haha really him) that using DMP after ethanol/acetone dehydration that tissue dehydrated well. We were working with the unicellular macro seaweed *Caulerpa*. I've since used it for fish scales. I can use 12-24 hour "instant dehydration" with DMP followed by two rinses with acetone before starting infiltration. Ellen Lavoie lavoie@uw.edu

I used this in diagnostic work in the 80s but stopped when I changed over to a university service lab. It can work fine depending on sample type. However, especially on cell cultures, I found it to be too harsh and the membranes did not stain well. When using hardy samples it is worth a try. But if using on an unknown sample or cell pellets be more cautious. Lou Ann Miller turtlelam@comcast.net

Thank you for your interesting posts. Use some caution with regard to the specimens being processed. When comparing morphology/staining /semithin sections /staining ultrathin sections with both techniques (traditional versus rapid dehydration), the latter can save many hours. I nevertheless recommend understanding how this works (in terms of chemistry and possible reactions/effects on specific tissue components that might be eluted or precipitated as usually found after dehydration with an ascending ethanol series). Wolfgang Muss wij.muss@aon.at

Epon Resin Embedding of Cell Monolayer

Microscopy Listserver

I am working on a correlative light and electron microscopy (CLEM) project. I have processed cells grown on an ibidi imaging dish for TEM using Epon (LX112) resin. However, the dish is not coming off the polymerized block, even after submerging the dish in LN_2. Can anyone suggest how to strip the sample block from the ibidi dish? ***Ravi Thakkar ravi.thakkar369@gmail.com***

I like to score the top with a single-edge razor blade and then slowly immerse the dish into liquid nitrogen. Not sure if scoring helps but it usually works for me. Slow seems better than fast immersion. Thomas Phillips phillipst@missouri.edu

I have not had success with ibidi dishes, but Mattek dishes are consistently good for Epon detachment. With some care you do not need to use LN_2 with Mattek dishes. Aleksandr Mironov aleksandr.mironov@manchester.ac.uk

I don't know what the dishes from Ibidi are made of but, with the classic polystyrene dishes (PS) or multi-well plates I also had a hard time detaching the Epon from the plate (flat embedding). I developed a trick involving a 2-step embedding protocol, pliers, and the application of brute force, but even then some pieces of PS stuck to the Epon block. One must be extra careful when cutting the block to first approach with a glass knife to remove the PS and upon reaching Epon switch to a diamond knife. Although I could get results with this method, it is imperfect, tedious and one needs to prepare the same sample 3 or 4 times to be sure to get 1 good block. But then again nobody said that research is easy. Stephane Nizet nizets2@yahoo.com

Are you using ibidi glassware micro-dishes and micro-slides with a glass coverslip bottom for high-resolution microscopy? In former days we used either Teflon® spray to put a fine 'separation'-layer on a glass slide/glass (or Thermanox)-"chamber" to aid the process of removing the epoxy resin 'flat' embedded sample for cutting, or the "heat and LN_2-aided" 'Pop(p)-Off' technique. In my own experience, there are some glass qualities which interfere with the process of detaching polymerized resin from the surface even using LN_2-cooling and abrupt warming to RT. For some hints see Muss, *Microscopy Today* (1998) https://doi.org/10.1017/S1551929500066773. Wolfgang Muss wij.muss@aon.at

The trick is to take the sample out of the oven before the Epon becomes fully hardened. With petri dishes, you can just hit them with a hammer then peel off the epoxy cast from the plastic. We published this as a Nature Protocol. Carol Heckman heckman@bgsu.edu

Help with OsO_4

Microscopy Listserver

I am preparing a TEM biological sample. I fixed trachea tissue using 2% OsO_4 after fixation by 2.5% glutaraldehyde and 4% paraformaldehyde. The color of the OsO_4 fixation solution should be colorless or light yellow, but became purple after 2-hours of OsO_4 fixation. Can anyone tell me what the reasons for the color change are?

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I would be very grateful for the kind help! Thanks, ***Jiawen Chen jchen124@stevens.edu***

The reason for the color change is residual aldehydes. Try rinsing again with the buffer solution and add more OsO_4. Hope that helps! Brittany Cymes bacymes@gmail.com

Maybe the lipids have leached out? Did you do normal rinses of the primary fix? Ellen Lavoie lavoie@uw.edu

Sounds like the osmium is old, make fresh. Geoff McAuliffe mcauliff@rwjms.rutgers.edu

I too have never had OsO_4 turn purple except when it was not fresh or compromised. Was the vial intact when opening? Sometimes OsO_4 will turn slightly yellowish, or at the most very light gray while processing tissue. The only time I have seen purple was when the osmium wasn't fresh. At this point you might try a new batch, but because of the delay of the post you have probably proceeded with processing. Have you been able to look at the tissue yet? Does the tissue look uniformly dark especially after ethanol dehydration? If the tissue is black, then things may have worked out after all. Won't know until you look in the TEM. Lita Duraine litaduraine@earthlink.net

I agree with Brittany that the most likely culprit is residual aldehydes. I have also had this happen with other chemicals that osmium tetroxide will react with, like TCH. Erin Stempinski stempins@ohsu.edu

Could it be that the trachea samples are covered on the lumen side by some mucus, some parts of which may come into solution and react with OsO_4? Perhaps you could replace the first osmium solution after 2h with a fresh one and see if the second incubation also leads to darkening of the solution? Stephane Nizet nizets2@yahoo.com

I've had that happen with samples containing lipids, fats, mucus, etc. The solution clears with subsequent buffer rinses before dehydration. Even if it continues further along, it should decrease with subsequent reagent changes. If it has lots of lipids/mucus in it, it may have a 'smeared' appearance even after being baked into resin. Take it all the way into resin as planned, section and stain it. I've also noticed samples will darken slowly, even after cacodylate buffer rinses. Terry Smith smithtl3@vcu.edu

Thank you very much for your advice and insight on my post. The solution became purple, not the stock solution. I washed the tissue samples three times using phosphate buffer after primary fixation. Then I used fresh OsO_4 to do the secondary fixation. After OsO_4 fixation, the tissues look uniformly black, but the fixation solution became purple. And it began to change color at about 1 hour after incubation with OsO_4. I will check using TEM. Hopefully the result will be fine. As Brittany and Erin pointed out, I will wash more times after primary fixation to remove residual aldehydes as much as I can for future experiments. Jiawen Chen jchen124@stevens.edu

Imaging with Expansion Microscopy

Confocal Listserver

We are currently starting expansion microscopy and would like to image structures that are ∼25 nm apart in Z (along the optical axis). After a 4x expansion structures will be ∼100 nm apart. So, my question is, what is the best imaging method for an expanded sample that provides ∼100 nm resolution in Z. It seems like people often do localization microscopy or sometimes STED on expanded samples, but I haven't seen many reports dealing with structures along the optical axis. My current thinking is iSIM might do the trick, but I wanted to ask the experts on this list as well. Thanks, ***Jeff Spector jospector@gmail.com***

100 nm is still too small for iSIM. 3D localization microscopy can do this in principle (there may be difficulty in imaging the relevant features dependent on sample structure and labelling). Alistair Curd a.curd@leeds.ac.uk

I agree with Alistair, 100 nm is still too small for iSIM. You may indeed try 3D STED on the expanded sample, or dSTORM, but in the latter case consider that you need to re-embed the gel in an uncharged gel and this causes a 20% loss in expansion, so you will have approximately 3X expansion. Davide Bambarotto davide.gambarotto@epfl.ch

Also, to complicate the measurements, in the few times I've imaged expanded samples it is not clear that expansion is isotropic, at least on a larger scale. As a Nobel winner wrote, "It was gravity which pulled us down / And destiny which broke us apart." The first phrase applies. And there may be apparent size difference in Z due to refractive index mismatch. Michael Cammer michael.cammer@med.nyu.edu

A few months back I helped someone who wanted to attempt expansion microscopy (ExM) (5x or so) on our Abberior Instruments STED. It worked and we got some nice, well-resolved images out of it, but there were some observations: (1) The sample was never "static" but either contracting or expanding. (2) Use a water immersion lens, at least for most ExM protocols (refractive index matching). (3) Be cautious of working distance! Keep your sample close to the coverslip, and don't start too thick, which may result in the ROI being outside what the objective can reach. (4) The sample was dim, probably because the expansion caused the fluorophores to be few and far apart. But on the plus side: great contrast, as virtually no background! (5) One benefit of STED is that it provides immediate feedback, so it is easy to see what you are doing in real time. (6) 100 nm resolution is easy to do with STED, and not too hard with 3D STED. You don't need a super high-power laser, assuming you use a good dye for it. Nicolai Urban nicolai.urban@mpfi.org

I would like to comment on some points that Nicolai mentioned and give some tips that might help. (1) True, but there are several ways to keep the gel steady. The one that I use is to coat the coverslip with PolyLysine and remove the water from the gel by using tissue, before placing it on the coverslip. This works well and the gel does not move at all. (2) True, but for single layer cells oil is ok. Of course, using a water objective is better. (4) Nicolai probably used a pre-expansion labeling protocol. In that case yes, since proteases were used to get rid of the sample inside the gel, many fluorophores are also lost. Moreover, fluorescence is diluted during expansion and linkage error is also 'expanded'. To overcome these issues, use a post-labeling protocol. Much much better! With Paul Guichard and Virginie Hamel (University of Geneva), we wrote a couple of book chapters to explain our UExM protocol in detail along with very useful tips and an explanation as to why post-expansion labeling can be more beneficial than pre-expansion labeling protocols. https://www.sciencedirect.com/science/article/pii/S0091679X20301242?via%3Dihub https://www.sciencedirect.com/science/article/pii/S0091679X2030145X. Davide Bambarotto davide.gambarotto@epfl.ch

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Strange Ice Pattern in Grid

3D Listserver

We have seen some strange ice patterns in our micrographs from K3-like attachments. One is grain-shaped ice, and another is wave-shaped ice, and some areas contain both. We obtained a tilt series and made tomograms, finding that the strange patterns only exist in the bottom of the ice layer and do not go through. Has anyone seen this before and know how these patterns form? I believe there are no problems with our grid recycle and storage system. ***Yutong Song aaabey@126.com***

I see this from time to time. It is contamination that occurred during plunge-freezing, during clipping of the grid on the holder, or during introduction to the microscope. I would suspect some cooling issues or deposition of non-vitreous ice after plunge-freezing. I don't know your setup. Make sure you have enough nitrogen evaporation during plunge-freezing and clipping of the grid on the holder is tight enough. Another possibility: are your samples in pure water or are you using organic solvents (such as DMF, THF, for instance)? Sylvain Trepout sylvain.trepout@curie.fr

The buffer is PBS, but there may be some formaldehyde and sucrose residue even after purification. Yutong Song aaabey@126.com

I think the wave is leopard-skin contamination, where the sample in the EM is not cold enough. Water sublimates and recrystallizes nearby. The other type could be residues from the grids. Did you try changing to another grid type? Henning Stahlberg henning.stahlberg@epfl.ch

Even in the same hole some areas contain the wave-shaped ice and some areas do not. While collecting data, temperature in the TEM is okay so I am not sure that the ice is affected by EM temperature. I use Quantifoil R1.2/1.3 Cu 300 mesh grids from the same batch. Trying other grid types is a good idea. Yutong Song aaabey@126.com

Indeed, as Henning stated, it is "Leopard ice". Are you using a side entry holder? It could be the clip ring holding down the grid is not tight, or the inner spring needs to be replaced. We also had issues with our Gatan 914 holder with Leopard ice that was only solved by having the holder serviced and the tip replaced. If it is an autoloader system, indeed it could be faulty clipping problems. Sharon Wolf sharon.wolf@weizmann.ac.il

Refining Polished Particles

3D Listserver

Dear all, can we use a 4-angstrom reference map with Relion 4 during refinement of polished particles? Also, can we do local alignment/refinement only if the particles before polishing are 2.5 angstroms (each unfiltered half map is 3.5 angstroms). Will this create a model bias? Any suggestions or thoughts are appreciated. ***Jay Rai jrai@fsu.edu***

In my experience, this should work okay if the initial angular sampling is such that the algorithm will move to finer sampling twice (or more), making sure that the refinement "settles in" properly. That said, I rarely do that, and if I do, I use slightly lower initial resolution. Basil Greber basilgreber@gmx.net

The half-sets are still separated in polishing, so iterative overfitting will be avoided. Just use the particles (data.star) from Refine3D and the postprocessing STAR file from your best refinement. The higher the resolution, the better, but 4Å will also do. Sjors Scheres scheres@mrc-lmb.cam.ac.uk

Advice on Relion/cryoSPARC Workstation

3D Listserver

I'm looking for advice on building a workstation for general compute tasks as well as cryo-EM data analysis (for example with Relion or cryoSPARC). The workstation will be placed next to a NAS storage device in our Data Center and few people will have access (less than 10). The informatics team will deal with installation and maintenance. Are there any minimal and/or optimal requirements, especially regarding GPU and RAM recommendation? I am completely ignorant on this matter, so I appreciate any advice! Best regards. ***Artemis Kosta akosta@imm.cnrs.fr***

There are many things to consider, and you should consult with an IT expert for the details. But the big picture is as follows:

- You will need 4 GPUs if you want to use cryoSPARC Live in a way that it can keep up with data collection (it assigns two GPUs for motion correction, CTF estimation and particle picking, then one GPU for 2D classification, then the 4th GPU for 3D reconstruction and refinement; if you have less than 4 GPUs, you need to stop one of these tasks to be able to start another, so it is less automated).
- The model of GPU you choose will pretty much dictate which case you need (some cases have enough room for only two of the latest GPUs). A common choice a couple years ago was the 4 Nvidia RTX 2080 Ti. I don't know about the latest models, but the successor of this one should offer good performance for cryoEM data processing. In any case, you want the "blower" type cards that exhaust the generated heat out of the case (as opposed to the regular type that exhausts heat inside the case, which puts more strain on the case's cooling system).
- Don't be cheap with RAM; 128 GB is an absolute minimum, and more will be helpful (not all programs for cryoEM data analysis can use a SSD scratch disk).
- Some job types in RELION are not GPU-accelerated (motion correction if you don't use MotionCor2, Bayesian polishing, CTF refinement), so more CPU cores is beneficial for these job types. If you face a compromise between less but faster CPU cores and vice versa, it is almost certainly more beneficial to choose slightly slower CPU cores, but more of them.
- You absolutely need a dedicated SSD scratch disk. The operating system (OS) is typically on an SSD in these workstations, so some people use their home directory as scratch space; but I would recommend against doing this! if not managed well, this will eventually fill this disk, and you don't want to run out of space on the disk housing the OS. The heavy read/write operations on the scratch space will also wear out the underlying disk faster, so again, not a great idea for the disk housing the OS: in case of disk failure, it is a lot easier to change a separate, dedicated scratch disk than reinstalling the OS and all programs.
- You absolutely need RAID6 for the workstation's internal storage (or a 10 Gb/s link to the NAS, and the NAS setup in RAID6); this is the sweet spot between hardware fault tolerance and read/write performance. A slow storage space will be limiting for the initial step of motion correction, where whole movies need to be read fast (and there are often too many movies to put them all on the scratch disk). I am using two systems, one with a 2-disk RAID1 and another with a RAID6 (forgot how many disks it has), and the difference in performance is very noticeable. On the RAID1 system,

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most of the time during a motion correction job is spent with the GPUs waiting for the disks; on the RAID6 system, the GPUs are all running at full capacity during the entire job. And don't be cheap with total usable storage space: having to move datasets in and out of the workstation too often because of limited storage space is quickly annoying. I would say no less than 40 TB, but of course this depends on how many datasets you typically work on at any given time and how large each individual dataset is.

- These guidelines are meant to optimize for performance, and a workstation of this type will likely be very noisy; but it should not be a problem if there is dedicated space for it. Guillaume Gaullier guillaume.gaullier@icm.uu.se

To add a few comments, based on my experience. GPU: we have systems with 2 and 4 2080 Ti, and both are used for image processing. If you go for cryoSPARC live, the more GPUs the better for parallelization, as Guillaume pointed out. Also, depending on available funding and the time you want to wait, you might not go for the very latest NVIDIA GPUs, as they are hard to obtain. This will most likely delay your order. Disk space: Our machines only have 20 TB, and we're constantly at a limit, with "just" 3-4 users. So, 40 TB is a very good starting point. SSD: We're running cryoSPARC without SSD, so I cannot state the advantages with this. But due to read/write speed of a SSD (compared to a HDD), I would also suggest a SSD as scratch disk. Christian Tueting christian.tueting@biochemtech.uni-halle.de

Towards the GPU question: From the MSRP the 3090 is probably the best option. It provides 24GB of VRAM, which is more than enough and a bit future proof. The single precision (FP32) performance is great, though it takes quite a lot of power. The problem is you will hardly get one for the MSRP and it will likely be double the price, and as far as I know there are nearly no blower style editions available of the RTX30-XX generation. Nvidia Founder Editions are not blower style since the 20th generation and custom board partners did not make or sell a lot of blower styles for the 30th generation. Easier to get these days are workstation/data center grade GPUs, though they are of course more expensive and not really needed. The A6000 for example is equal or a bit better than the 3090 in FP32, though significantly more expensive. A Data Center alternative would be the A40, which is a passive cooled system, so only for servers with high airflow. Kilian Schnelle kilian.schnelle@uni-osnabrueck.de

I want to add a note about SSD space, as I didn't see it among the excellent advice provided: make sure that your system is using PCI-E enabled SSDs (these are commonly denoted as M.2/NVME in traditional desktop systems, or U.2 in server-style systems). But regardless, you want to avoid having SSDs that are SATA or SAS because they will be rate-limited by the communication bus. PCI-E SSDs are typically an order of magnitude faster. This adds an additional consideration, as well, which is PCI-E lanes of the CPUs and/or chipset. Traditional desktop class CPUs like AMD Ryzen or Intel i-series have substantially fewer PCI-E lanes, and this limits SSD expansion possibilities, especially if using 4 GPUs, which will also all eat up PCI-E lanes. Workstation classes such as Threadripper, Threadripper Pro, and Xeon, and server classes such as Epyc and Xeon, will typically offer far more PCI-E lanes. Adding a 2nd CPU to a system adds even more PCI-E lanes. A general rule of thumb is you should expect each GPU to use 8 lanes, and each PCI-E SSD to use 4. Colin Gauvin colingauvin@montana.edu

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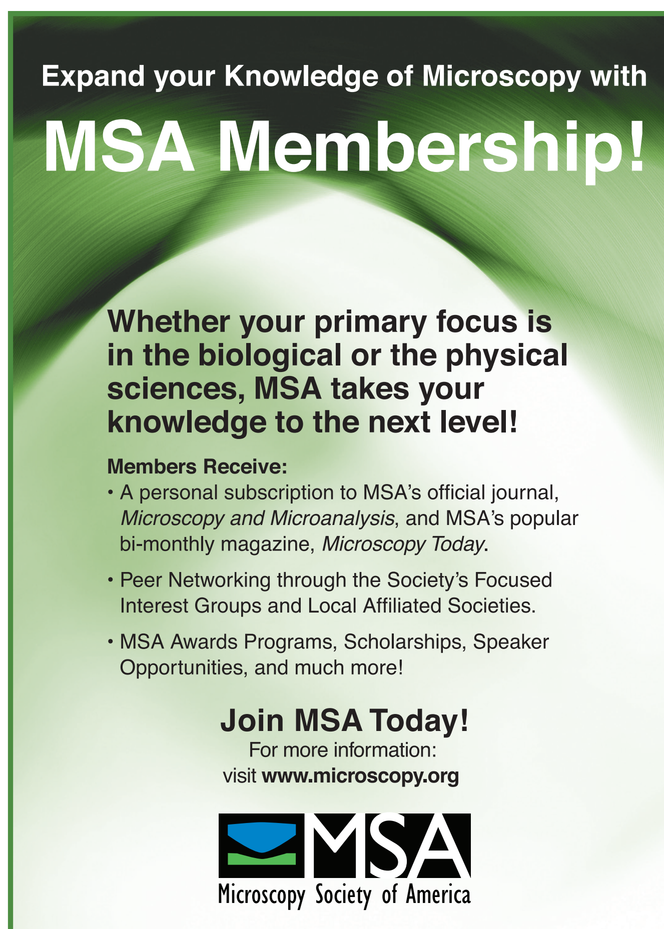

Crossword Puzzle Answers

See puzzle on page 54.

1 Z	2 A	L	3 U	4 Z	5 E	C	■	6 M	I	7 C	8 H	9 A	10 E	11 L
12 E	M	■	13 G	E	L	■	14 U	C	■	15 H	O	W	I	E
16 I	I	■	■	17 R	O	18 S	S	■	19 H	O	A	R	S	E
20 S	C	■	21 O	N	D	R	E	22 J	■	R	■	23 Y	W	U
24 S	I	25 M	■	26 I	E	■	27 D	U	28 R	E	29 R	■	30 E	W
■	■	31 O	32 S	K	A	33 R	■	34 L	E	■	35 U	X	I	E
36 H	37 O	O	K	E	■	38 I	B	E	A	39 M	S	■	40 N	N
41 E	R	N	I	■	42 G	G	■	43 P	R	A	K	44 A	S	H
45 N	B	■	46 N	47 A	■	48 S	49 O	S	■	50 R	A	Y	■	O
51 D	I	52 C	K	E	53 Y	■	H	■	54 A	B	■	■	55 W	E
56 E	T	A	S	■	I	■	57 M	U	L	L	58 E	59 R	■	K
R	■	N	■	60 D	M	61 T	S	■	62 L	E	N	A	■	■
63 S	64 T	E	65 M	M	E	R	■	66 E	A	■	■	67 G	A	68 P
69 O	B	L	I	V	I	O	70 N	■	71 R	72 E	73 N	U	■	A
74 N	I	E	L	S	■	75 G	O	L	D	S	T	E	I	N