

Many of these tips were learned at the Carl Zeiss Microscopy Superresolution Imaging Class which I attended in June 2017. Others have come from reading, on-site training with Zeiss personnel, and experience.

The managers of a superresolution core facility at Harvard University have said:

Superresolution microscopy (SRM) “is a technique that can greatly reward the rigorous user but has the potential to punish the casual user. To achieve optimal and reproducible results, SRM requires careful planning of specimen preparation, rigorous attention to instrument optimization and image acquisition, and a thorough understanding of the practical limitations, sources of error, and artifacts.”

Navigating challenges in the application of superresolution microscopy, Talley J. Lambert, Jennifer C. Waters
J Cell Biol Dec 2016, jcb.201610011; DOI: 10.1083/jcb.201610011

Image acquisition tips

Warmup time

The instrument (excepting the lasers, fluorescence lamp, and the PC) is left on all the time to keep the temperature stable. After a complaint about sample drift, our service engineer is recommending that the lasers and the fluorescent lamp be turned on two hours ahead of time. We can show you how to accomplish this without accumulating an additional 2hrs of billed time on the instrument.

Samples, oils, etc. should all be temperature equilibrated to avoid specimen drift and temperature-related refractive index (RI) issues. Since the inside of the Elyra’s laser box is a few degrees F warmer than the room, you should put the oil and your samples inside the chamber to equilibrate them before using them.

Optimal image greyscale levels for acquisition

Ideally each image will have a greyscale histogram that shows it using between 3000-5000 grey levels when imaged using the SIM grid. Less than this will typically lead to image data that shows artifacts after processing. More than this is unnecessary, as this will likely increase acquisition time. Also, after processing the images will have a much larger histogram (between 40,000-65,500 greyscales) and you do not want to saturate your image intensity data by starting out with too bright raw data. Your goal is always good signal-to-noise in the images (which is why non-specific background can be a real problem).

If you have an exceptionally bright artifact in the image, you may want to move it out of the field of view. If you set the greyscale intensities with the artifact in the field, you may end up with the intensities in your areas of research interest that have insufficient grey levels to SIM process correctly.

Optimizing image settings to keep acquisition time manageable

Choose to increase the laser power in each channel first, rather than increasing the camera exposure settings for a given channel. This will keep image capture times for the entire Z-stack shorter.

If your blue channel is just DAPI

If you are doing a multicolor z-stack and the 405 channel is just DAPI to show the location of the nucleus (assuming no particular need for superresolution), set the z sampling rate (step size) using the 488 line instead, this will save a few image steps in a large stack. The software sets the z sampling rate based on the shortest wavelength used, with shorter wavelengths having the smallest step thickness.

Sometimes when imaging DAPI the signal is so strong that it is difficult to see the grid in the sample image using the blue channel. Since the post-processing software expects to remove the image of a grid from the sample, this can cause problems. To fix this, you can use a SIM grid one level more coarse (giving up a bit of resolution) to ensure that there is a grid visible in the raw sample images.

Three vs five rotations?

- If your sample tends to bleach quickly, try 3 rotations (note that if there is more than a 30% drop in intensity between the first and last rotation in an image plane there will be image processing artifacts). If your sample is fading this quickly, the fluorophore/mounting media combination may be unsuitable for SIM.
- If using non-hardening media with the possibility of things moving, use 3 rotations.
- If you are looking at structures that are primarily bright spots, you can probably do 3 rotations.
- If you are looking at things that are longer and have directionality (e.g., cytoskeleton), then 5 rotations will avoid potential artifacts. The directionality issue can become really problematic when the rotation angle lines up with the direction of a filament, as it can cause the image to radically drop in intensity, but with 5 rotation angles you can compensate for that better than when using 3 rotations.

Single image plane SIM

To get the full 2x resolution improvement in the central image plane, you need a minimum of 5 Z slices. The plane of interest, and then two images above, and two images below that slice at the appropriate step size.

Capture smaller area

You can acquire smaller areas (FOV) using a smaller portion of the camera chip.

Thicker sample trick

In a thick, scattering sample try using more coarse grid spacings for all of your channels than what the system recommends. You will lose some SIM resolution, but you should be able to image a little deeper. Refractive index mismatch and spherical aberration cause the grid to blur out at depth. If the image processing software cannot “see” the grid in the images, artifacts are created when the software tries to remove it.

Optimal samples

Overall the Elyra works best with cultured cells on a coverslip or tissue sections 10um (or less) thick. Ideally samples are fixed and transparent (clearing techniques can help), with a very good signal to noise (S/N) ratio. A good discussion of optimal samples can be read here: <http://aicblog.janelia.org/?p=264>

There are no shortcuts for sample prep in SIM. We encourage you to read and re-read the [Sample Preparation Guidelines for Superresolution Structured Illumination \(SR-SIM\)](#) as well as the handout on [#1.5H high performance coverslips](#) found here: <http://microscopy.arizona.edu/information-regarding-elyra-s1>

If you are using a hardening mounting media, you must wait the full time for it to harden (often a week) to get the optimal refractive index (RI) of the media. Thermo-Fisher has a relatively new mounting media, Prolong-Glass, which has nearly the same RI as glass.

Samples approaching 20um in thickness would probably be better imaged using the Zeiss LSM880 confocal/multiphoton with Airyscan detector located at the UA's Imaging Cores – Marley. Airyscan can give up to a 1.9x resolution improvement over standard confocal. <http://microscopy.arizona.edu/equipment/zeiss-lsm880-nlo-upright-multiphoton>

Image processing tips

Reduce SIM processing time

If possible, crop the raw image data before processing to speed things up. You can skip a step by drawing the box on your data, selecting the image dataset for SIM and then telling it to process, without having to crop first. Still you might want to crop & save anyway, for data purposes.

Channel alignment

Any/every time the Elyra's hardware changes (changing filter cubes, cleaning/removing objectives, service visit) we need to do a channel alignment calibration using a bead slide. Users would be wise to include some 100nm four-color beads (<https://www.thermofisher.com/order/catalog/product/T7279>) either in their sample or mounted on a slide using the same conditions as their sample to ensure that their channel alignment calibration is correct for the conditions found in their sample. An observation shared at the Zeiss class: It's all well and good that the core manager can resolve test samples (4 color 100nm beads, GATTAquant DNA origami), but there is no guarantee that users, with different sample preps & mounting media, will obtain the same resolution. *Note – the GATTAquant rulers need to be flat (orthogonal) to the image plane to get the correct measurements.* Still, at least you know the system is performing at spec...

Batch processing

Samples can be set up in the software such that a batch processing run will be performed on a single file folder. You can tell the workstation what you want done, walk away and come back the next day. Out of courtesy to other users, please reserve the time on the SIM workstation in iLab. The workstation time is currently billed at a rate of \$0.00/hr (or, at no cost).

NOTE: batch processed files are given a file extension of OUT. We know from experience that the ZEN Black software can open the OUT files, but the ZEN Blue software cannot. The solution is to open the OUT files in the ZEN Black software and then re-save them as standard Zeiss CZI files, which can then be opened in the ZEN Blue software.

Manual processing tips

The assumption here is that users will first SIM process their images using the automatic settings. In the information tab for the processed images there is a log showing how things were processed, with numbers that can (if needed) be changed a bit in manual processing. The log is long, so it can be handy to paste it into Windows WordPad or Notepad and read it there (*select all using "control A" and then copy using "control C", paste into an empty WordPad document using "control V"*).

- Auto-noise filter (Wiener) – you can increase this number a little if you have good signal-to-noise in your image. This will add back in some higher frequency information, but do not get too aggressive. The typical values range from -2 to -6. If you are going to modify the Weiner filter, the "peak/mean" value (see the info) needs to be above 10.
- Sectioning – if you have issues with the grids showing up in the images post-processing, you can increase these values to 100 (all three of them).
- Recommendation – use the raw scale to avoid the images being normalized (which is what happens in automatic mode). Baseline cut will rest the lowest value to black, which you may (or may not) want.
- Good news – after you change the settings the way you like them, choose to reprocess the already processed images. This will speed things up, because some of the preliminary processing is already done. Use Combine and make sure to save with a new file name!

How much data should I keep?

We haven't sorted that out yet, and there's not much guidance online. We do not expect to have the hard disk space on the processing workstation to store user data for more than 2-4 weeks at most. If your lab expects to be a heavy user of the Elyra, we encourage you to plan for the ability to store as much as several terabytes worth of data. Please remember that once you have transferred your data from the Elyra computer to the processing workstation, you should delete the data that remains on the Elyra computer.

There are several types of data:

- Raw data (CZI) - Typically these are quite large, with the file size dependent on the number of fluorochromes imaged, rotations used, and z-stack thickness. Raw data for image stacks can range from 5-20GB per stack. You want to keep the raw data, since you always re-process the data using different settings if needed. If you crop your raw data before SIM processing, you may want to save the cropped raw data.
- SIM processed data (CZI) - After processing the data file becomes much smaller, but still in the 0.5-5GB range. Because SIM processing takes time, you may want to keep this data as well.
- Channel aligned images of SIM processed data (CZI) - These files will be the same size as the SIM processed data. We encourage users to keep a copy with their data of the *.BIN alignment file (1kb in size) that was used for this step. This will allow you to reprocess your SIM data and then re-do the channel alignment at a later date. Please make sure that you leave the original BIN files on the workstation hard drive.
- Exported TIFF images or videos - Since the free ZEN software does not run on Macintosh computers, sometimes it's easier to export the files to TIF, or use the power of the workstation to create a video of a rendering. Please delete these as soon as possible after you are done with them.

Users are responsible for their own data.

Transferring files

We recommend that labs purchase a USB 3.0 external hard disk drive for file transfer (USB 2.0 is too slow!). Because of the size of the RAW data CZI files, the hard disk should be formatted as NTFS. This is because Windows computers cannot read the native Macintosh hard disk format and the Windows hard disk formats called FAT and FAT32 cannot handle the large CZI files that the instrument creates. The same formatting issues hold true for USB flash drives.

Both the Elyra computer and the workstation have a blue USB 3.0 compatible cable that you can use to attach your hard dish or flash drive to the computer.

Getting ZEN for a lab computer

There should be an installation file for ZEN on the workstation computer that you can copy. Down at the bottom of the webpage (see URL) are instructions for installing the ZEN software on a Windows (7, 8, 8.1, 10) computer (to avoid all the extra software drivers), as well as some other ZEN related instructions & tips: <http://microscopy.arizona.edu/learn/printable-materials>

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From the [UA Microscopy Alliance](http://microscopy.arizona.edu/learn/printable-materials) - <http://microscopy.arizona.edu/learn/printable-materials>
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