Sample Preparation Guidelines for Superresolution Structured Illumination (SR-SIM)

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Definitions

Refractive index (RI) – (Wikipedia) In optics, the refractive index or index of refraction of a material is a dimensionless number that describes how light propagates through that medium.

- Refraction of Light - https://micro.magnet.fsu.edu/primer/java/scienceopticsu/refraction/index.html
- Refraction of Light - https://micro.magnet.fsu.edu/optics/lightandcolor/refraction.html

Chromatic aberration – (Wikipedia) In optics, chromatic aberration (abbreviated CA; also called chromatic distortion and spherochromatism) is an effect resulting from dispersion in which there is a failure of a lens to focus all colors to the same convergence point.

- Chromatic Aberration - https://micro.magnet.fsu.edu/primer/java/aberrations/chromatic/index.html
- Common Optical Defects in Lens Systems (Aberrations) - https://micro.magnet.fsu.edu/primer/lightandcolor/opticalaberrations.html

Spherical aberration – (Molecular Expressions) The most serious of the monochromatic defects that occurs with microscope objectives, spherical aberration, causes the specimen image to appear hazy or blurred and slightly out of focus. The effect of spherical aberration manifests itself in two ways: the center remains more in focus than the edges of the image and the intensity of the edges falls relative to that of the center. This defect appears in both on-axis and off-axis image points.

- Spherical Aberration - https://micro.magnet.fsu.edu/primer/java/aberrations/spherical/index.html
- Focus Depth and Spherical Aberration - https://micro.magnet.fsu.edu/primer/java/aberrations/pointspreadaberration/index.html

Fluorophore Selection

The SR-SIM is equipped with 405, 488, 561, and 642 laser lines. All traditional fluorophores (GFP, mCherry, Alexa dyes, Cy dyes, Mitotracker, DAPI, etc.) are compatible with the system. Care should be taken to maximize the brightness of the fluorophores, either by testing expression levels or antibody staining protocols. Selection of robust fluorophores is strongly recommended. In general, if a sample appears dim or is easily bleached by a traditional wide field or confocal system, SR-SIM will be limited in its ability to reconstruct a superresolution image.

Ideally the fluorophore emission should be matched to fit nicely within the range of the filters (Elyra FS Flex, 000000-2264-374) that we have on Elyra S.1 at the University of Arizona.

Position	3		4		5		6	
CUBE	DAPI center	DAPI width	GFP center	GFP width	RFP center	RFP width	CY5 center	Cy5 width
Position 3 - QUAD	450	53	524	48	597	43	690	61
Position 4 - DAPI/GFP/CY5	450	48	525	40			730	140
Position 5 - GFP/RFP			524	48	597	43		
Position 6 - DAPI/CY5	450	53					690	61
DICHROIC emission specs	*450*	*40*	*523*	*35*	*599*	*33*	*693*	*55*

Currently there are two configurations for each lens:

- ***QUAD Cube*** *- DAPI, GFP, RFP, CY5 using the same filter position, in theory this should speed up acquisitions a little, with the caveat that there could be some spectral bleed-through*
- ***4 separate cubes*** *(DAPI = Pos3, GFP = Pos 4, RFP = Pos 5, CY5 = Pos 6) more like what we had before, one cube/channel*

Quantum yields *(the ratio of photons absorbed to photons emitted through fluorescence)* for:

- AlexaFluor dyes: http://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook/tables/fluorescence-quantum-yields-and-lifetimes-for-alexa-fluor-dyes.html
- Cyanine dyes: https://biotium.com/technology/cf-dyes/ *or* https://www.apexbt.com/probes-dyes/cyanine-dyes.html
- Fluorescent proteins: http://www.microscopyu.com/articles/livecellimaging/fpintro.html

Sample Thickness

Thicker samples will provide more of a challenge for SR-SIM imaging. The instrument can be expected to work well with sample thicknesses of up to 5 to 10 microns. Deeper imaging is possible, depending on the refractive properties of the sample, the intensity of the fluorophores, and how heavily the sample scatters light. Please be aware that the deeper you attempt to image, the more difficult it will be to achieve a superresolution image.

Use of Proper Coverslips

Most ZEISS oil or water lenses are designed to image through 170 micron thick coverslips. Most commercially available coverslips can range in thickness up to 12%. The upper end of this can cause a severe reduction in resolution. To avoid this, Carl Zeiss (and others) now produce high performance coverslips which vary in thickness by no more than 2.9%. Use of these coverslips is strongly recommended for preparing samples for either traditional fluorescence imaging or superresolution. Sources of #1.5H coverslips, culture dishes, and chambers: http://microscopy.arizona.edu/sites/default/files/sites/default/files/upload/coverslips_for_microscopy.pdf

Use at most only two coverslips per microscope slide. Do not have the coverslips flush with the edge of the microscope slide. The microscope slide needs to fit into a piezo insert and will not fit if the coverslips are fixed near the edges.

The Elyra S.1 stage insert can hold a single 35mm culture dish. The dish must have a #1.5H coverslip bottom to correctly image using SIM. If the cells will still be in culture media, then the 63X water/glycerin objective lens is most likely the only option for imaging. Unless the sample is very flat (thin in the z plane), there will be spherical aberration issues with using an oil immersion objective lens.

The Elyra S.1 stage insert can hold a coverslip (#1.5H) bottomed chamber, similar to the *Nunc™ Lab-Tek™ II Chambered Coverglass*. Be careful to only order chambers with the correct coverslip thickness. The same issues regarding objective lens choice apply as with the 35mm dishes.

Use of Proper Mounting Media

Mounting media should be used that has a refractive index (RI) as close as possible to the coverslip (1.52). The mounting medium and the immersion medium should be matched within 0.01–0.05* ideally to three decimal places** (*B. Foster, 2003; **J. Pawley, 1997, Archives of the Confocal Listserv).

We have successfully used the following products:

- Vectashield Mounting Medium from Vector Labs (Item Number H-1000), which has a refractive index of 1.44, works well for SR-SIM. This is a non-hardening media so you must be sure to completely seal the coverslip with something like nail polish. Antifade properties of the several varieties of Vectashield:

https://vectorlabs.com/products/all-products/mounting-media/vectashield-antifade-mounting-media-if.html check these for their dye compatibilities.

- Prolong Gold (P36930) from Thermo has a higher refractive index, 1.46, but this is after 160 hours of curing. It must be cured for at least 60 hours to reach a refractive index of 1.44. As it is a hardening media, you may also notice it can somewhat flatten samples. Thermo offers this information about the antifade properties of the Prolong Glass, Gold, and Diamond mounting media (compared to the hardening version of Vectashield) https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cellular-imaging/fluorescence-microscopy-and-immunofluorescence-if/mounting-medium-antifades/prolong-gold-antifade.html
- Prolong Glass (Thermo P36982) is a fairly recent addition (2018). The mounting media has a refractive index of 1.52 after curing (approx. 48hrs). This refractive index is almost identical to that of glass. Based on data provided by Thermo, users should see some improvement in axial (z) resolution and a slight improvement in lateral (XY) resolution (since SR-SIM does not image very deep, the gains from the refractive index match are more moderate). https://www.thermofisher.com/order/catalog/product/P36984?SID=srch-srp-P36984
- Fluoro-gel, with TES buffer (17985-31) from Electron Microscopy Sciences has a refractive index perfectly matched to glass at 1.52. This is also a hardening media and must be allowed to cure for the correct length of time. Although it has the highest refractive index, and makes it ideal for imaging, it will also harden even more so than the Prolong Gold. As such, it may cause flattening of samples. Information: https://www.emsdiasum.com/fluoro-gel-a-direct-substitute-for-biomeda-gel-mount

In general, other mounting media can certainly be used, but take care of the refractive index. As the closer the refractive index is to glass, the more likely it is to flatten samples, it may be required to test which mounting media is best for the samples. Do not use mounting media with DAPI mixed in; this can cause general autofluorescence which will interfere with superresolution imaging.

Common problems resulting from mounting media include:

- Mowiol – because the user mixes this mounting media in their lab, the refractive index of this mounting media can be a problem. Insufficient mixing can mean that the mounting media is non-homogeneous, causing image distortions. The refractive index will be slightly different with each batch.
- Washes - Not removing the wash media sufficiently prior to mounting. This can mix with the mounting media and cause changes in the refractive index or problems in hardening. Mounting samples that still have residual water can cause variability in the refractive index of the mounting media because of incomplete mixing.
- Sealing the coverslip - Not properly sealing the coverslip if the mounting media is non-hardening. The mounting media can leak out and mix with the oil. Nail polish can be used to completely seal the coverslip. Nail polish must be completely dry before imaging slides on the Elyra. Wet nail polish can damage lenses. *Please note there are anecdotal reports that sealing coverslips with nail polish can quench fluorescent proteins. Others have said that they do not experience this issue. Users should test their own samples.*
- Curing time - Not allowing a hardening mounting media to completely cure. Artifacts will occur from imaging into a sample with a lower refractive index. Refer to the manufacturer's instructions for how long a given mounting media must cure.
- Bubbles - Trapping bubbles in the mounting media. Bubbles can refract light and cause artifacts.

- Bleaching – Because SIM imaging requires high laser powers, samples need to have an anti-fade in the mounting media. To prevent artifacts, it is important to avoid more than approximately a 30% loss in intensity between the image rotation positions. Users can assess this by watching the maximum greyscale intensity on the camera over time.

Other sample prep considerations:

- Cleanliness - Excess mounting media, fingernail polish, oil from a different brand of microscope, or fingerprints on the coverslip will adversely affect the imaging. Please make sure the coverslip is scrupulously clean before bringing your sample to the SIM microscope.

- Mounting sectioned tissue - With all of the superresolution methods, the closer the sample is to the coverslip, the better the images are. In a telephone conversation with Mr. Chhun we asked if sectioned tissue needed to be mounted on the coverslip or if it could be mounted on the slide. He indicated that it could be mounted on the slide, IF the layer of mounting media between the top of the section and the coverslip was "thin". If you choose to try mounting sections on the coverslip - please be aware that #1.5H coverslips are not typically sold as coated or "charged". This means that the sections could float off during immuno-staining. There are a number of ways to improve "stickiness", including a thin coating of poly-L-lysine.

- Note: in a 2015 webinar presentation, the company that makes the OMX (a competitor's SIM instrument) strongly urged that sections be mounted on the coverslip, since it is very difficult to reproducibly control the thickness of the layer of mounting media between the coverslip and the sectioned tissue.

- Chamber slides - We do not recommend the use of chambers that are used to grow cells directly on a microscope slide and include a non-removable gasket. The distance that the gasket creates between the cells and the coverslip when the slide is assembled will not allow for good SIM imaging due to spherical aberration problems. The best way to image cells using SIM is for the cells to be grown directly on a #1.5H coverslip.

- Effect of temperature on immersion oil refractive index - the refractive index of immersion medium will vary with temperature approximately 0.0004 per 1°C (per Cargille Labs); the variance will be even more pronounced with wavelength. Per Zeiss' recommendations, we use a 37°C immersion oil. The chamber on the microscope is several degrees (F) warmer than the room, we highly recommend giving your samples at least 1hr to equilibrate to the microscope temperature prior to imaging as thermal issues can cause movement, which reduces the microscope's ability to acquire super resolution images.

What types of samples will work best with SIM?

To achieve the enhanced resolution of SIM several phases of patterned light are projected into the sample at different angles, with either 15 images (3 rotations) or 25 images (5 rotations) per image plane. After the images are acquired, the post-processing is somewhat similar to deconvolution microscopy. The data is processed using Fourier math, the higher resolution information is extracted from the moiré fringe information, the grid patterns removed, and then a new image is created. While Fourier math is a powerful image processing tool (MRI imaging and other signal processing disciplines use this extensively), there are known issues and artifacts.

Discrete structures like cytoskeletal filaments, membrane-bound labelling, and other small bright localizations of fluorescence (e.g., cytoplasmic organelles) will image best. A strong signal to noise ratio will provide good images.

Diffuse fluorescent labelling does not post-process well and is prone to image processing artifacts. This short blog post from the HHMI Janelia Farms website explains the issues well, see: *"Structured-Illumination Microscopy: What types of sample are not suitable and why"* at https://www.aicjanelia.org/post/samples-for-sim

Strengths

- 2D and 3D imaging
- co-localization at high resolution
- morphology
- improved image contrast

Weaknesses

- live cell imaging is very slow, if not impossible *(~1 image plane/second depending on sample brightness and number of colors being imaged)*
- inability to compare relative fluorescence intensities
- diffuse fluorescence staining is prone to image processing artifacts
- thicker samples (>15 um) are more difficult to successfully image at superresolution

This document was originally created by Bryant Chunn (Carl Zeiss Microscopy) in approximately 2013. It was added to in 2016 and revised in 2018 and 2023 (new filter set, updated URLs) by Douglas Cromey. Mr. Cromey's additions are in green text, and it is not implied that Carl Zeiss Microscopy endorses or supports Mr. Cromey's additional material.

A number of the included tips & pointers were gleaned from materials found online at:

- Light Microscopy Core Facility, Duke University - http://microscopy.duke.edu/
- Mounting Media and Antifade reagents - Tony Collins, Wright Cell Imaging Facility, Toronto Western Research Institute, University Health Network - https://bidc.ucsf.edu/sites/g/files/tkssra806/f/wysiwyg/Mountants_WCIF.pdf
- BiteSizebio presentation – Super Resolution Imaging - SIM Capabilities, Sample Prep & Analysis, Patrina Pellett, PhD, GE Healthcare, Cell Analysis, May 6, 2015 - https://bitesizebio.com/webinar/practical-super-resolution-imaging-sim-capabilities-sample-prep-and-analysis/
- Advanced Imaging Center at Janelia Farms (HHMI) - Lin Shao, Ph.D. - https://www.aicjanelia.org/post/samples-for-sim

Mr. Cromey is grateful for the support he receives from the SWEHSC (P30-ES006694) and RII.