SIM technical notes:

The N-SIM is a commercial implementation of the instrument described in the following papers:

Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. M. G. L. Gustafsson Journal of Microscopy (2000) 198, Issue 2, 82-87.

Three-Dimensional Resolution Doubling in Wide-Field Fluorescence Microscopy by Structured Illumination BioPhys J: M. G. L. Gustafsson Volume 94, Issue 12, 15 June 2008, Pages 4957–4970

Subdiffraction multicolor imaging of the nuclear periphery with 3D structured illumination microscopy. Science. 2008 Jun 6;320(5881):1332-6

2D Vs 3D SIM

2D SIM only works for very thin samples – bacteria, yeast or in vitro type samples. For optimum resolution (laterally) the best option is to do TIRF-SIM since the spacing of the pattern is such that you get the best possible resolution enhancement. You should also only be creating an evanescent wave of around 100 nm so it is a very thin illumination.

The 2D-SIM grating only projects the pattern in 2D and is appropriate for samples which literally comprise of the Cartesian coordinates x and y only. As we know all Eukaryotic cells are three dimensional. With the 3D grating the moire pattern becomes a checkerboard type effect. It also uses a slightly different configuration of laser light beam which enables collection of light from slightly above and below the 2D plane. This fills in any minima in the fourier domain and is important because SIM image processing occurs on a fourier transform of the original image. Importantly one region which gives a minima when fourier transformed in 2D is the centre of the sample, as biologists this is where we image, which gives you patterns laterally and axially. So the advantage of using the 3D SIM is you get better resulting contrast and Moire fringes on samples where the fluorescence is coming from different planes. This requires 15 images and not 9, but comes with the advantage / requirement that all of the light from the plane of the sample you are imaging is taken into account when carrying out SIM processing.

3D SIM comes in two different modalities. 3D Stack – which is the original Gustaffson implementation of 3D SIM and 3D Slice – a newer implementation which can speed up imaging but there is a tradeoff.

3D Stack: This is the original 3 beam SIM using the 0 +1 and -1 order of light as described in the 2007 Biophys J paper. It requires 3D data through at least 1um axially and z spacing must be 120nm to fulfil the requirements of the equation from the 2007 paper in 3D. However providing this is met the system will generate a high quality 120nm resolution image.

3D Slice: Nikon offer a 3D SIM slice option. This comes with a background subtraction algorithm similar to that described here: https://onlinelibrary.wiley.com/doi/full/10.1111/jmi.12259 (Although not the same, the principle is the same). The slice option pre-processed the raw images and removes the background before the SIM processing. This means that it is no longer necessary to image through 1um axially to obtain a super-resolved image. Instead 1 axial plane is sufficient – providing the data are processed using the 3D Slice option. The disadvantage is the slide option subtracts both the signal and the noise from the raw image and this needs to be carefully carried out. In samples where there is poor modulation of signal – i.e. the stripes from the grating are rubbish – then using Slice processing will degrade the final resolution of the image. So the output image quality and resolution may well suffer. Slice option tends to give slightly less well resolved images – typically 130 – 180nm. If the sample is really well stained or is clean Slice results can be comparable to or better than Stack. It just depends. That said if you want to acquire an image without a Z stack and time is important 3D Slice is a great way to do imaging.

SAMPLE PREPARATION:

Plating cells

SIM sample preparation in many respects is similar to sample preparation for publication quality confocal imaging. Since SIM prefers flat samples using coverslips which are made to high precision is recommended. High Precision coverslips:

ZEISS High precision Cover Glasses 18mm^2 order Number:474030-9010-000

Marienfeld Superior coverslips: www.marienfeld-superior.com

18mm^2 (Cat.No. 0107032). 22mm^2 (Cat.No. 0107052) Round 18 mm diameter (Cat.No. 0117580).

UK resellers CellPath Ltd UK High performance coverslips no 1.5H 18x18mm (Cat.No SAN-5018-03A)

For glass-bottom petri dishes and multi-well plates see:

- Willco (http://www.willcowells.com/)
- Lab-tek (offered through Thermo)
- MatTek (http://glass-bottom-dishes.co

Immunofluorescent staining

All antibodies / dye staining should be optimised so background is minimised. For preference Fab2 fragment secondary antibodies or Nanobodies (chromotek) should be used. Diffuse staining or staining with a high background will cause poor image reconstruction. Any dyes or fluorophores which bleach will also cause poor image reconstruction, eGFP and mCherry are not ideal for these applications for this reason.

Mounting samples:

The sample should be embedded in a medium matching refractive index (RI) of the immersion oil (n=1,514).

Buy in solutions

- Fluoromount/ Fluoroshield (Sigma) RI of 1,40. F4680-25ML (£50 ish)
- ProLong Gold (Life Technologies). Be aware this embedding medium needs 2 day to harden (in order to reach a constant RI). In addition your sample may shrink during this process. Refractive index (RI) of the cured product: 1,46.
- SlowFade Gold (Life Technologies) with RI of 1,42.

DIY solutions (Not tested at time of writing)

- 86% Glycerol with 2.5g/l DABCO (1,4-deazabicyclo[2.2.2] octane, Sigma Cat. (D2522) in Tris-HCl. 1M pH 8.0.
- 2,2'-thiodiethanol (TDE) – aqueous. RI can be varied ranging from being that of water (1.33) to that of immersion oil (1.52) by appropriately diluting with water.

System operation best practise.

- The system needs to warm to 27 and stabilise degrees before data can be acquired. Temperature changes can induce artefacts in the system so please let Super-res facility staff know if the system is not at 27^{o}C.
    - We therefore ask people to only switch off the lasers and nothing else when they finish. Otherwise the system needs to heat up and stabilise for 3 – 4 hours prior to imaging.

- When acquiring data good image contrast is important. This means optimising staining protocols. When looking live it should be possible to see the grating moving in the raw images this will look like twinkling.
- Clean lenses particularly important with SIM. Anything that helps improve signal to noise, minimises aberrations (particularly spherical) and leads to good stripey pattern generation will help get the best out of your system. Other things that degrade performance in the real world are:- dirty coverslips, low quality coverslips with variable glass thickness across them, loose sample holders, bent sample holders and generally badly mounted samples.
- Aim for high signal to noise but relatively short exposure times ideally. The longer your exposure time the more chance you have for vibration-induced artefacts

- An image of the 3 rotations of the gratings should have equal frequency modulation in all 3 gratings. If you don't see this please find the super-res facility staff immediately
- To acquire a true 3D SIM image it is necessary to acquire a focal series which is about a micron thick. This means you shouldn't need as much high resolution noise suppression in your reconstruction as well.
- Take care not to knock the system when acquiring data. The anti-vibration table will compensate for any other vibrations

SIM Artefact – Summary table

• **Artifact type**	**Example**	**Possible cause**	**Troubleshooting**
High frequency noise (hammerstroke pattern)	Image 1	1.Low modulation contrast due to low contrast of the structure of interest 2.High out of focus blur contribution 3.Weak sample intensity 4.Dynamic range too low 5.High resolution noise suppression parameter set too low	*Sample* 1.Choose suitable structure for labeling 2.Use brighter/more stable dyes 3. Reduce background by nonspecific labeling and avoid autofluorescence *Acquisition* 1.Increase dynamic range by increasing preferably laser power but also exposure time until photobleaching is limiting 2.Reduce size of 3D z stack but ensure in 1st and last planes are just out of focus (no stripe pattern should be visible) *System* 1.Use camera mode with higher photon well depth 2.Decrease gain 3.Use lower readout speed *Reconstruction* 1.Increase value of high resolution noise suppression parameter
Refractive index mismatch artifacts (doublings/ghosting of structures along z axis)	Image 2	1.Mismatch between the assumed optical response of the system encoded in the OTF and the effective optical response in the sample. *Sample* 1.Wrong coverslip thickness 2.Sample far from coverslip surface 3.Correction collar of objective set to wrong position-varies with temperature and sample	*Sample* Match optical properties of the sample and the OTF *Acquisition* Adjust lens correction collar to correct for imaging depth and/or temperature
		System	Run grating calibration utility

		Grating position- distance to relay lens	
Six-point hexagonal repeat (honeycomb pattern)	Image 3	1.Out of focus blur contribution to image 2.Low frequency contribution is missing from axial direction (missing cone problem)	*Acquisition* 1.Reduce contribution of out of focus light by shallow z-stack acquisition *Reconstruction* 1.Use stack reconstruction algorithm 2.Increase value of out of focus blur suppression parameter
Channel mis-alignment	Image 4	Chromatic aberration in x, y or Z	*Can be caused by issue with piezo and the Chromatic correction being incorrect. Ask facility to recalibrate instrument.*
Z-wrapping- Orthogonal course stripes and decreased contrast /resolution throughout reconstructed dataset. Prominent echo signals of opposing z-ends	Image 5	First/last frame of z stack starts/ends in prominent in-focus sample structure. 3D-SIM algorithm loops information from last to first slice to allow the Fourier processing to work (assumes infinite structures).	*Acquisition* Extend height of z stack such that the structure of interest is just out of focus in the first and last image planes (no stripes visible in raw image)

Image 1 – high frequency noise

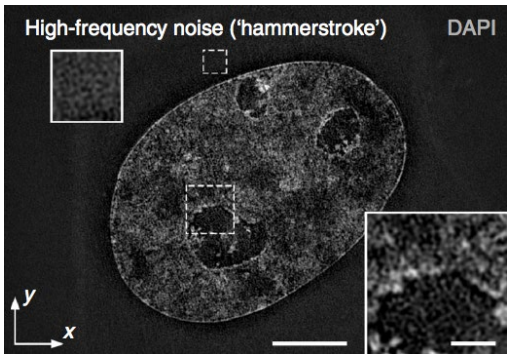

Image 2 – Refractive index mismatch

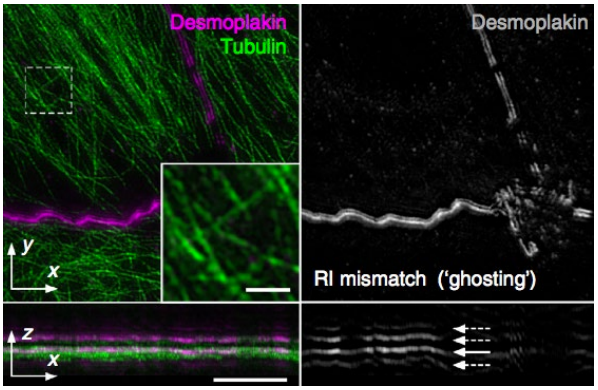

Image 3 – Hexagons of doom.

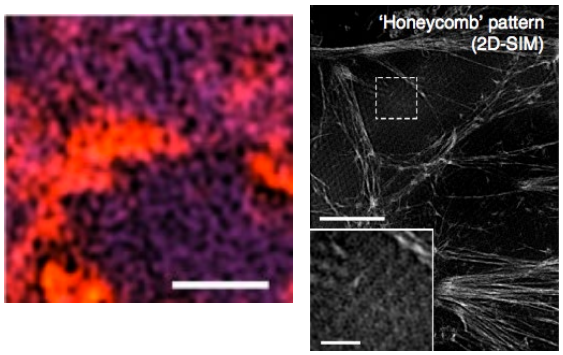

Image 4: Chromatic mis alignment in X,Y or Z

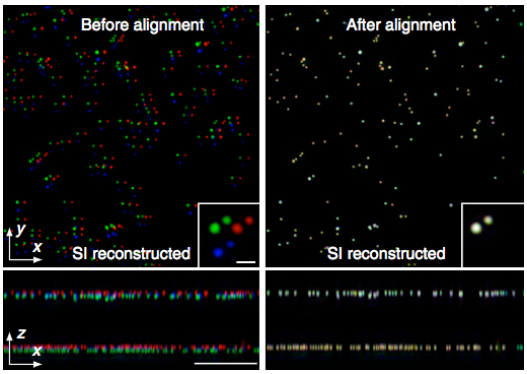

Image 5 – weird effect caused by insufficient information in the z axis