Sample prep:

- 1.5 thickness coverslips are best for SIM

- We recommend Prolong Gold/Diamond or Slowfade Gold (not Vectashield) for mounting media. In our experience this will maximize resolution, however curing mounting media that solidifies may compress your sample in height. (You may also use homemade polyvinyl alcohol mounting media such as Gelvatol or Mowiol.)

- TIRF SIM has to be conducted in aqueous media (e.g. PBS, or FluoroBrite for live cells)

Imaging:

- Use 100x objective only for SIM with Nikon immersion oil type NF
    - FOV = ~70 µm x 70 µm (~4x larger than with previous camera)
        - You can use the ROI option to image with the same FOV as previously
    - Maximum imaging depth:
        - 3D-SIM: ≤ 7 µm
        - 2D-SIM: ≤ 3 µm
        - TIRF-SIM ≤ 200 nm
- Single camera imaging: double click the single camera NIS Elements icon
    - Do not turn on Camera "1" if you are not doing dual
    - Make sure "Hamamatsu with N-SIM" is selected on startup
    - Do not use exposure times <"1 frame"; this equals ~13ms (~3x faster than previous camera)
        - When acquiring smaller fields of view in "ROI" mode, do not use exposure times <20ms (this mode recalculate what "1 frame" equals)
- Dual camera imaging: double click the dual camera NIS Elements icon
    - Make sure Camera "1" is turned on and dual-cam unit is set-up and aligned (ask Josh or Dina for help with this)
    - Make sure "Hamamatsu with N-SIM" is selected on startup
    - In dual-camera mode, do not use "1 frame", use exposure times >20ms
        - Only green (525/50 em.) and red (605/70 em.) can be used with dual-cam mode
            - Use only the "eyes" optical configurations, and the "488 561 Dual" OC
        - There will be a small amount of green to red bleed-through in dual-cam mode
            - Talk to David for help with bleed-through corrections if needed. To estimate the exact bleed through you need a single stained control (green fluorophore only)
- Do not go above ¼ of the camera dynamic range, <~16,000 on the LUTs window

- o Normally you will get great reconstructions with MUCH lower LUT levels (e.g. 1000)
- If you are doing a z-stack and want best axial resolution use 120nm step size, and Stack Reconstruction. If you use the Slice Reconstruction, or the Thumbnail view for reconstruction, use 200nm step size (the step size recommended for the objective)
- Chromatic aberrations may cause channel alignment issues is XY and Z, particularly with thicker samples and towards the edges of the field of view. To determine the shift in xy and z we need multi-color beads, ideally mounted in the same way as your sample.
  - o Talk to David for help with channel alignments.

Image reconstruction:

There are three different parameters for reconstruction with 3D SIM, which generally have to be determined empirically:

- **Illumination Modulation Contrast**: should usually be between 0.5-1
  - o "Auto" may also be used
- **High Resolution Noise Suppression**: should generally be <1
  - o The higher it is the fewer artefacts you will have, but also the lower the resolution
- **Out of Focus Blur Suppression**: usually 0.1-0.2
  - o The higher it is the less blur, and resolution, you will have
  - o This is only used in 3D SIM, not 2D or TIRF SIM
- Tick the box to use separate settings for each channel
- Do not tick the box "Deconvolve when Illumination Contrast is Low"
- After reconstruction, look at FFT, which should be a high contrast six lobed shape without lots of dots/points evident, and then adjust LUTs on reconstructed image, find a small spot and use the FWHM function in the Intensity Profile tool to estimate resolution
- Do not use the "Image Registration" module in the SIM Panel - Talk to David for help with channel alignments