fairSIM quickstart guide

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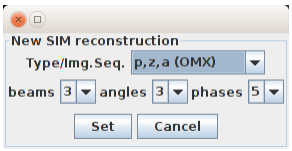
This manual provides a short, step-by-step guide on running a SIM reconstruction by fairSIM, parameter-setting and trouble-shooting tips, and a description of our test dataset. **Update:** Some features marked *(development version)* are only available when using the current development release of fairSIM, not the originally published version of our code.

Contents

1 Step-by-step guide

For datasets of adequate quality, fairSIM offers a largely automated reconstruction mode, relying as little as possible on user input. The steps are also labeled in the main GUI (see fig. 1).

- **Open the raw images.** ImageJ / FIJI will read most microscopy files via BioFormats. Please use the *split channels* function (either at import or through FIJI), so that each channel appears as a separate stack / window.
- **Start fairSIM**. Choose "new reconstruction" from the plug-in menu, optionally enabling (verbose) output of the reconstruction process to the ImageJ log. Alternatively, an existing set of reconstruction parameters can be loaded.
- **Specify basic parameters** For a new reconstruction, the first window asks for basic parameters: 2- or 3-beam illumination (2 or 3 bands), the number of angles / pattern orientations used, and the number of phases for each orientation. *By selecting either OMX or Zeiss, these parameters are set automatically.* Also, the ordering of the input image stack (phases, angles, z-planes) is set accordingly.



- **1 - Import the image stack**. In fairSIMs main window, image selector, choose a raw data stack and click *select* (clicking the list refreshes available images). A window opens and allows to:
  - Select the slice to reconstruct, optionally from a wide-field projection of each slice. Choose a slice with enough structure well in focus.
  - Set (or override) the physical pixel size.
  - Subtract a constant camera background offset.

  After a successful import, the image selector shows the currently imported image name in green. Additionally, a window displays the raw input data from the selected slice. For 3-beam illumination, the coarse pattern should clearly be visible on these images.
- **1b - Select time-lapse reconstruction** *(development version)* In the "Image selector" field, a second "Timelapse" tab allows for reconstructing time series of SIM images semi-automatically. The "z-slices" input has to be set to the number of slices acquired for every time-step, the total number of time-points is determined automatically. Once a reconstruction has been completed for one time-step, batch reconstruction and an automatically updated reconstruction result (recomputed when moving the time slides) becomes available.
- **1c - Image Corrections** *(development version)* The third "Correction" tab allows for a basic (global) correction of varying intensities between different SIM angles and phases. Variations are estimated by comparing either averages or medians of the raw images. Large variations should be cross-checked, as they point to problems in either instrument alignment or sample properties (check with a known-good sample, e.g. a bead slice, when in doubt).
- **2 - Load or approximate the OTF**. An optical transfer function can either be loaded from file [1] or approximated. When choosing *approximate*, the numerical aperture of the objective and the emission wavelength have to be entered. An additional parameter allows to set a deviation from the ideal OTF, and is set to a reasonable default.
- **3 - Run the parameter estimation**. For adequate data sets, the parameter estimation should work without fine-tuning. Clicking *run*, after some seconds a window will open and show a visual feedback of the fit process. Each slice represents one pattern orientation, and should contain a clearly visible peak found by the fitter (see fig. 2 for an example and explanations).
- Optionally: **4 - Switch on OTF attenuation for optical sectioning**.
  OTF attenuation is very helpful when performing single-slice reconstruction of 3-beam, 3D data. For the idea, see e.g. [1]: The 2D OTF is attenuated such that frequency components with poor axial resolution (missing cone) are suppressed and filled from other bands. The attenuation parameters are described in more detail in the 'Settings' section. To tweak them, start from the defaults and increase/decrease the 'strength' parameter first.

[1] OMX OTF files can be converted to our format with a small, additional plugin.

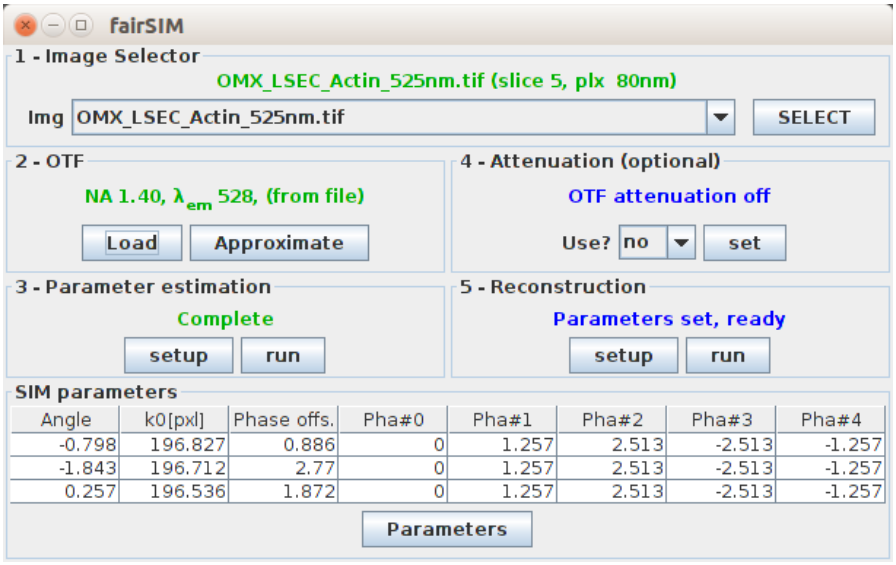


Figure 1: Screenshot of fairSIMs main GUI. The numbers label the different reconstructions steps, incomplete steps show up with red labels. Here, the first 3 steps have been completed, and the dataset it ready for reconstruction.

- **5 - Run the reconstruction**. After a few seconds, a new window shows the reconstruction result, and the wide-field image (with and without Wiener-filtering) for comparison. Parameters (Wiener filter, apodization cutoff) can be defined in *setup*.

  In the *(development version)* Richardson-Lucy (RL) filtering following [2] is available. The type of filtering used and the iteration count can be accessed via "setup".

  The *(development version)* version also allows to influence the shape of the apotization function $A(k)$ via the "APO bend" parameter b. Here 1.0 corresponds to a triangular shape, lower numbers push medium frequency response (roughly as $A(k) = (1-k)^b$ with $k = 0 \ldots 1$).

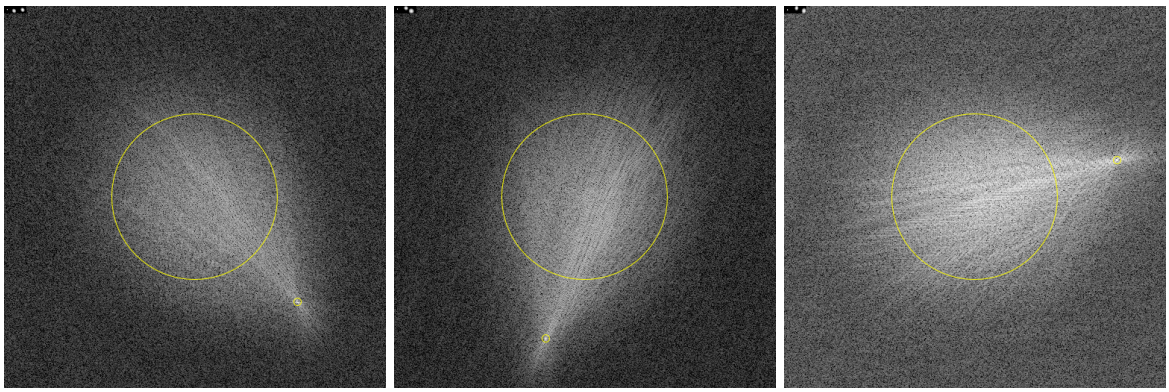


Figure 2: Power-spectrum visualization of the cross-correlation used to estimate reconstruction parameters, for three pattern orientations. The smaller circles mark where maxima in the correlation have been found and fitted, the large center circle marks the low-frequency regions excludes from peak fit (default at less than 0.6 of the OTF support, settable in the parameter estimation menu). The three insets on the top left visualize the results of the subsequent, iterative, sub-pixel precision fits. These should show clear elliptic, in most cases circular structure. **If instead the left inset shows noise**, this is a clear indicator for a failed fit.

2 Settings and troubleshooting

Both the parameter estimation and the reconstruction provide a 'setup' menu to influence the amount of intermediate result output and to provide access to further settings. FairSIM also supports additional functionality for advanced users, to assist in cases where more difficult input datasets are used.

2.1 Installing and running fairSIM

After downloading the plugin `.jar` file, fairSIM is installed either by copying the file to ImageJs/-FIJIs plugin folder, or by using the `plugin, install` feature. Using the menu-based install, some versions of FIJI display an error message (`WARNING: The PluginClassLoader cannot be reset`). This is a known bug in FIJI, after a restart of FIJI the plugin should nonetheless be available.

When successfully installed, fairSIM can be found in the `plugin` menu, typically towards the end of the list (as the name starts with a lowercase letter).

If fairSIM / Fiji hangs: In rare cases, fairSIM and/or Fiji hangs, typically after closing a fairSIM (intermediate) result window. This is caused by Fiji removing the image window while it is still in use by fairSIM. To avoid these problems, do not close result windows while fairSIM is active. We are investigating[2] ways to mitigate this problem without interfering with the result display.

2.2 Load / Save

The SIM reconstruction parameters (displayed by the table in the main GUI) can be saved to a file, optionally together with the OTF. The function can be accessed through the 'parameters' menu.

A reconstruction can be loaded from file when starting a reconstruction from the ImageJ menu.

Please note that currently, shift vectors are not converted to physical units, i.e. a parameter set for a 512×512 pixel input image will *not* work on a 1024×1024 image. If this poses a large drawback, please contact us.

[2] If you are familiar with Java development, and this error occurs, you can help us by sending in a stack-trace.

2.3 Input images

FairSIM works for reasonable input image sizes. The most common cases (512×512 and 1024×1024 pixels) are well tested, and are preferred. Images of arbitrary size will be zero-padded to form a square image, where the size is a multiple of 32. The outermost 10 pixels of all image borders are faded to zero, to avoid stray components in frequency space.

2.4 Troubleshooting the parameter estimation

If the steps described above do not yield a successful parameter estimation, some advanced settings are available. For users familiar with the SIM parameter estimation and reconstruction process, they can help to trouble-shoot the process.

The parameter estimation is a two-step process working on the band cross-correlation data: First, the peak in the cross-correlation is localized, to pixel precision, over the complete image. In a second step, this localization is iteratively improved to sub-pixel precision. If it fails, some steps can be taken:

- The peak localizer outputs the iterative sub-pixel fit results in the top left corner (see fig. 2), as 3 blocks of 10×10 pixels. Especially the first iteration should show a clear, noise-free point-like structure, as it covers ±2 pixels around the initial estimate. If this is not the case, the initial, coarse localization of the peak failed.
- The initial peak localization assumes the peak to lie beyond 0.6 of the maximal OTF support, i.e. at least a 1.6-fold resolution improvement. The limit can be adjusted from the GUI, either up (if stray components yield a wrong localization) or down (if the input data provides less resolution improvement, see our SLM-SIM test dataset).
- Increasing the amount of intermediate output provides spatial representation of the overlapping frequency regions used for the cross-correlation parameter estimation. These should be checked for information content (see fig. 3). If there is little structure in the overlapping regions (e.g. a rather featureless membrane stain), the cross-correlation has no information to work on.
- If this problem occurs for 3-beam data, the parameter estimation can be switched to work on the first instead of the second band. This makes the estimation less precise, but more robust, as the overlap region is much larger. If the sample shows a clear illumination pattern, but the parameter estimation fails, try this setting.
- The parameter estimation, as currently accessible through the GUI, assumes equi-distant phases. An auto-correlation based phase estimator for arbitrary phases is implemented[3], please contact us if you need GUI access to this feature.

2.5 Settings for the reconstruction

Wiener filter and apodization are set to reasonable defaults, but can be tweaked to obtain optimal results.

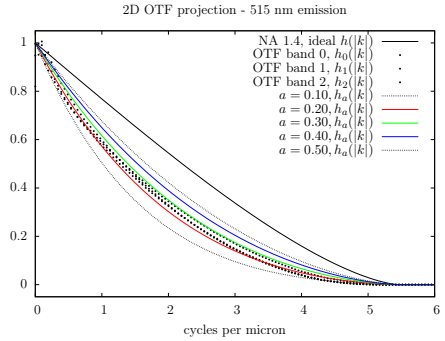
- Please note that the Wiener filter parameter will be squared, i.e. the GUI sets ω, not ω^2.
- The apodization cut-off is set in multiples of the OTF cutoff; for datasets where the resolution improvement deviates from $2\times$, the parameter can be adjusted.
- The output can be switched to three modes:
  - Raw output: Includes negative values and performs no scaling, useful for in-depth analysis
  - Clipped output: Negative values are clipped to zero, no further scaling is applied
  - Clipped and scaled output (default): Negative values are clipped to zero, the result is normalized to a $0\ldots255$ range (but kept at 32bit precision).
- For datasets of thick(er) samples, dialing in the optical sectioning through the OTF attenuation is often the most essential step of acquiring a high-quality reconstruction.
  - Test different settings, by varying the `strength` (typically $0.95\ldots0.999$) and `FWHM` (typically $0.8\ldots2.0$)).

- The `LSEC1...OMX` dataset provides a good example: The attenuation has little effect on the flat region (lower right side), but a large effect around the cells core, where there is more out-of-focus contribution.
- The Tetraspeck samples are rather flat, so the attenuation has less effect

2.6 Importing or estimating the OTF

An experimentally determined OTF is preferred. For users of the OMX microscopes, a converter tool is available to extract 2D OTFs from the OTF calibration data provided by the system. For other systems, a SIM OTF generation tool is in development.

Alternatively, a rough OTF estimate can be provided by fairSIM. This requires setting the NA and emission wavelength of the sample (keep in mind the NA might be reduced due to the immersion medium), and allows for setting a "dampening" parameter a to shift the medium frequency response of the OTF estimation. The lower a is set, the more medium frequencies are assumed to be dampened by the optical system. For the example data published from the OMX system, a has been determined to have a usable range of 0.3 to 0.5.



2.7 Setting the amount of intermediate output

Both the parameter estimation and the reconstruction step can output a variable amount of intermediate results.

For the parameter estimation, these are:

none — No intermediate results are outputted.

standard — A summary of the fit result for each pattern orientation is outputted, see fig. 2. This helps to quickly spot failed parameter fits, and is thus set as the default

most — Power spectra and spatial domain representation of the overlapping bands used in the cross-correlation analysis are outputted. For three-beam data, correlations are outputted for both the lower and the higher band, fit results are shown for the band selected for the fit.
This mode helps to spot if the overlapping, medium frequency regions contain enough information for a successful fit.

full — Power spectra of the raw, shifted bands are added to the output. Serious errors in the band separation should show up in these spectra, but otherwise the mode should not be needed.

For the reconstruction step, these are:

none — Only the final, reconstructed SR-SIM image is shown

standard — SR-SIM reconstruction, wide-field, wiener-filtered wide-field. This helps to easily compare the SIM reconstruction and the wide-field image.

more — Power spectra and spatial domain images of all pattern orientations and bands are added to the output. This visualizes the information gained through the SIM process, and also helps to spot errors in single pattern directions (uneven illumination, etc.).

most/full — Currently the same mode, *full* for further additions. Adds frequency domain representations of the optical transfer functions to the output. This helps to check if the OTFs fit the data.

3 Modifying, enhancing and automating fairSIM

Since fairSIM is open-source under GPL, it can freely be used, but also modified and extended for new purposes. To this end, the fairSIM source code features a modular design (see fig. 4) using Java packages:

- The `linalg` package provides 1, 2 and 3-dimensional vector objects (both real and complex valued) and implements basic linear algebra routines (additions, scaling). Fourier transformations are delegated to the `JTransforms` library, an open source project featuring a fast, multi-threaded, pure Java FFT implementation (similar to FFTW in C/C++).
- The `sim_algorithm` package implements the optical transfer functions, parameter estimations and the actual SIM reconstruction.
- The `sim_gui` package provides all components of the graphical user interface (see fig. 1).
- The `ImageJ/FIJI` package allows fairSIM to operate as an ImageJ plugin. It contains all functions to import images and display (intermediate and final) results.
- The `utils` package provides logging, configuration file access and routines for easy multi-threading.

The modular layout allows one to easily reuse fairSIMs components. Especially low- and high-level functionality of `sim_algorithm` can be used from scripts, without resorting to the GUI. The git repository includes an example file[3] , in which a complete reconstruction sequence (setup, coarse and fine parameter estimation, reconstruction) is run. This file is commented, and should be easy to adapt to e.g. automated batch data processing.

Also, the data structures and functionality in `linalg` can probably be employed to implement new reconstruction methods (e.g. the current deconvolution-based approaches), as these methods provide more convenience than working with pure Java data types.

Documentation JAVADOC comments have been used throughout the source code, especially to document public API functions. Thus, after downloading the source code, the API documentation can automatically be generated, either by invoking javadoc by hand or through the provided makefile.

[3] See https://github.com/fairSIM/fairSIM/blob/v1.0.2/org/fairsim/fiji/TestPlugin.java for the complete algorithm and https://github.com/fairSIM/fairSIM/blob/v1.0.2/org/fairsim/sim_algorithm/SimAlgorithm.java for ready-to-use high level functions.

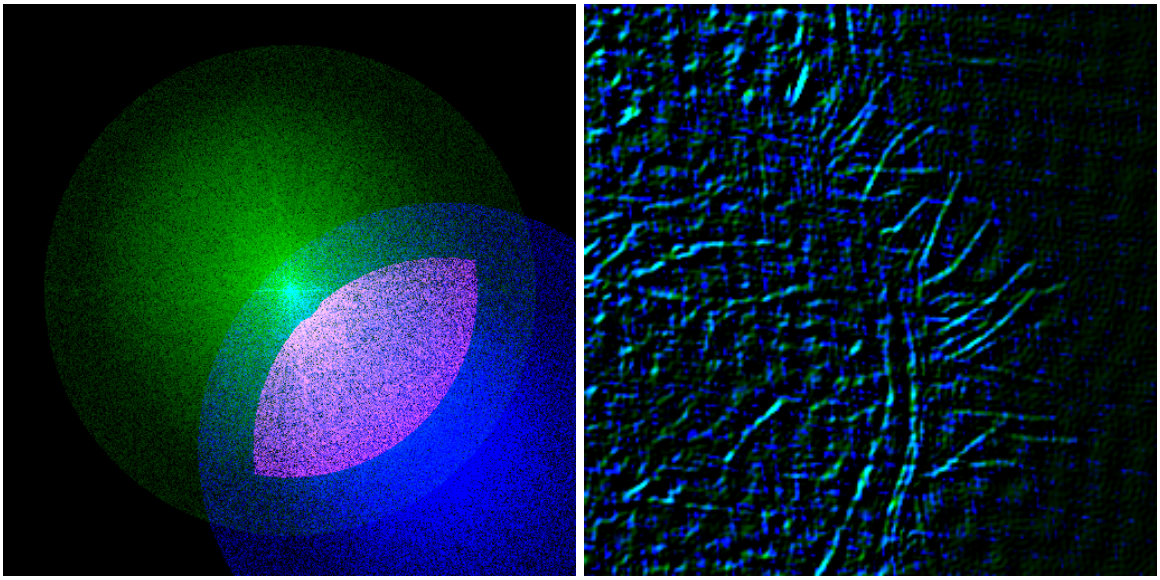


Figure 3: Common region of SIM bands 0 and 2 in a three-beam reconstruction. Left: Power spectra of band $\tilde{S}_0$ (green), shifted band $\tilde{S}_2$ (blue) and the common region (magenta), defined here by $h(\vec{k}), h(\vec{k}+\vec{p}) > 0.05$, i.e. both bands' OTFs are above a certain threshold. Right: Composite spatial representation of only frequencies from the common region, with S_0 (green) and S_2 (blue), base dataset U2OS actin. The spatial representation shows that enough information content is present in both bands to successfully fit a peak in the correlation.

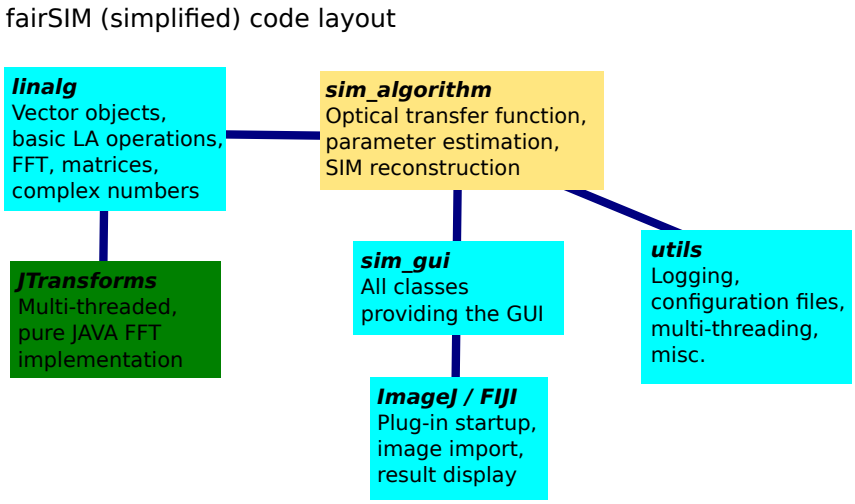


Figure 4: FairSIM modular source code structure

4 Test datasets

As an additional download, we provide the raw datasets used to produce the figures in our publication and the supplemental information. Here, the parameters used for these reconstructions are given, so they can serve as an easily accessible test:

4.1 OMX examples

The OMX uses 3-beam illumination, 3 angles, 5 phases, images are ordered *phases, z, angle*. This is automatically selected when choosing "OMX". The NA is 1.4, also set as default. The OTF can either be approximated or loaded from the provided file. The pixel size is 80 nm, it should be automatically set from the data file. The table below provides all further parameters needed:

File	$\sim \lambda_{\text{em.}}$	slice	Background	Att. str.	FWHM
`OMX_LSEC_Actin_525nm.tif`	525 nm	5	85	0.99	1.2
`OMX_LSEC_Membrane_680nm.tif`	680 nm	5	350	0.995	1.2
`OMX_Tetraspec200_680nm.tif`	680 nm	4	80	0.98	1.2
`OMX_U2OS_Actin_525nm.tif`[a]	525 nm	7	–	0.9995	2.0
`OMX_U2OS_Mitotracker_600nm.tif`[b]	600 nm	6	90	0.997	1.5
`OMX_U2OS_Tubulin_525nm.tif`	525 nm	4	140	0.999	1.2

[a] Good test dataset for optical sectioning
[b] Noisy dataset, set the Wiener filter to 0.09

4.2 Zeiss examples

The Zeiss Elyra uses 3-beam illumination, 5 angles, 5 phases, images are ordered *z, angles, phases*. This is automatically selected when choosing "Zeiss". The NA is 1.4, so the default value is correct. The pixel size is 64 nm, and should be automatically set by from data file. Files are provided both as 1024×1024 pixels full field-of-view, and as 512×512 pixels crops for faster analysis / testing. The table below provides all further parameters

File	$\sim \lambda_{\text{em.}}$	slice	Background	Att. str.	Att. FWHM
`Zeiss_Actin_525nm_*.tif`	525 nm	5	140	0.995	1.2
`Zeiss_Mito_600nm_*.tif`	600 nm	3	140	0.995	1.2

Zeiss example datasets courtesy of Marcus Behringer and Markus Sauer, University of Würzburg.

4.3 TIRF-SIM example

The TIRF SIM dataset was acquired for the work published in [4], thus courtesy of Peter Kner. The illumination parameters are 2-beam, 3 angles, 3 phases, the raw frames are in default order. Emission wavelength is approx. 525 nm, the objectives NA is 1.49. The images contain live-cell data captured at high frame rates, thus signal levels are somewhat lower (consider setting the Wiener filter to e.g. 0.1). It is also important to correct the rather high camera offset (background 405), and the region excluded from **parameter fitting has to be set to 0.7**. As a TIRF dataset, the optical section should of course be turned off.

4.4 SLM-SIM example

Our SLM-SIM example represents a typical dataset from the early stages of our microscope development. Its quality is not on par with the results from commercial microscopes. It is included to show that fairSIM can also handle datasets of simpler, home-build setups.

The SLM-SIM uses 2-beam illumination, with 4 angles and 3 phases, the images are ordered *phases, angles* (no *z*, as only one slice is captured). Use the default setting and input the parameters above accordingly. The NA is 1.2, the emission wavelength is 680 nm. For parameter estimation, the **fit region has to be adjusted**. As it defaults to a resolution improvement of 1.6×, it should be lowered to e.g. 0.4. The images have rather large camera background offset, so consider setting a background subtraction of 280 when importing the stack. Also, the apodization filter can be reduced to 1.6, as the full resolution enhancement is not reached.

References

[1] Wicker, K., Mandula, O., Best, G., Fiolka, R. & Heintzmann, R. Phase optimisation for structured illumination microscopy. *Optics express* **21**, 2032–2049 (2013).

[2] Perez, V., Chang, B.-J. & Stelzer, E. H. K. Optimal 2d-sim reconstruction by two filtering steps with richardson-lucy deconvolution. *Scientific reports* **6**, 37149 (2016).

[3] Wicker, K. Non-iterative determination of pattern phase in structured illumination microscopy using auto-correlations in fourier space. *Optics express* **21**, 24692–24701 (2013).

[4] Kner, P., Chhun, B. B., Griffis, E. R., Winoto, L. & Gustafsson, M. G. Super-resolution video microscopy of live cells by structured illumination. *Nature methods* **6**, 339–342 (2009).