

How to optimise eSRRF parameters for test datasets

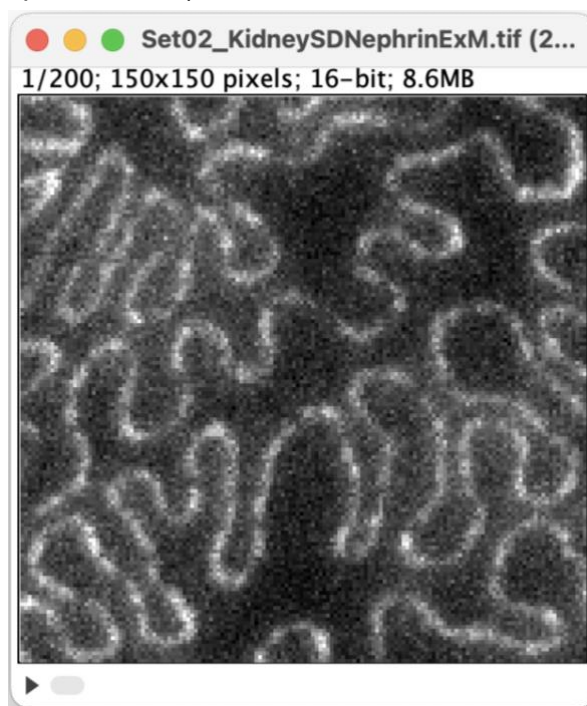
Chapter 1: eSRRF Parameter optimisation in Fiji

1.1 Software installation

The basic requirement is to have Fiji and the NanoJ-eSRRF plugin installed. Detailed instructions can be found on GitHub (<https://github.com/HenriquesLab/NanoJ-eSRRF/wiki>). The test datasets can be downloaded here: <https://zenodo.org/doi/10.5281/zenodo.11518140>.

1.2 Image loading and preparation

1. Start Fiji and open the image, in this case the example data set 02 will be used (Set02_KidneySDNephrinExM.tif).

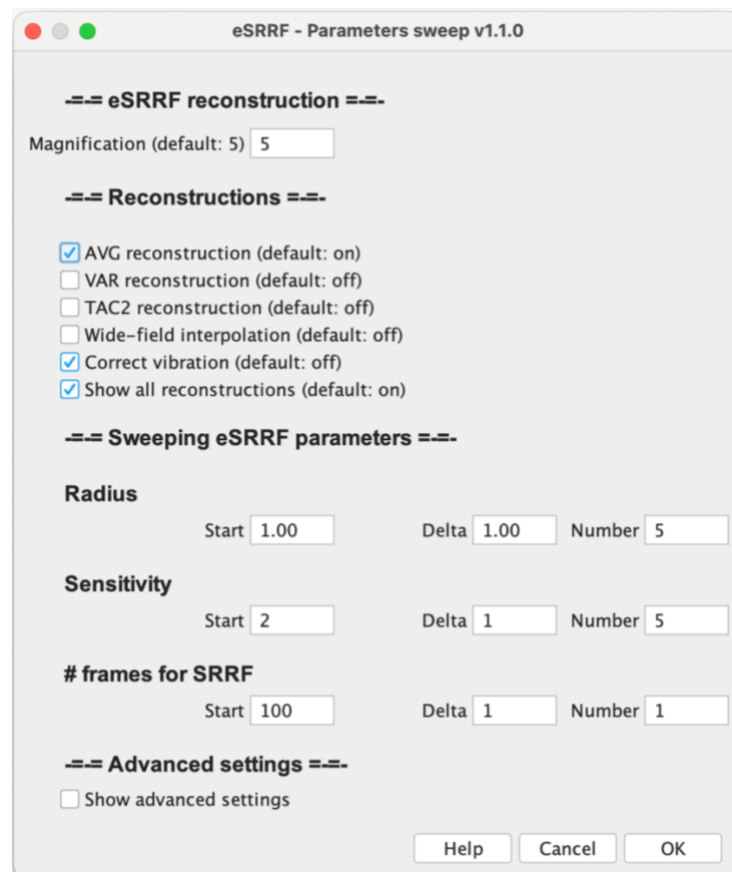


2. Set the correct pixel size in the image by clicking on properties and setting the correct dimensions and units (select “*Fiji → Image → Properties*” and input correct pixel size and units).
3. To run the parameter sweep, crop the image in a region with the signal of interest and background (e.g., one-quarter of the image).



1.3 Find the optimal set of parameters

1. Run the plugin “*Fiji* → *Plugins* → *NanoJ-eSRRF* → *eSRRF – Parameter Sweep*” with the following parameter settings in the main and advanced settings window:



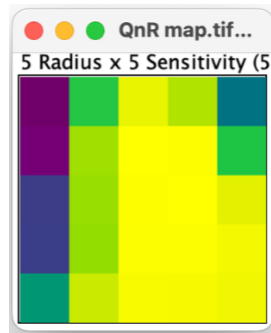
In this example, the Correct Vibration option is chosen to correct for the apparent vibration of the image. The output of this plugin is several stacks with eSRRF reconstructions for each parameter set, the corresponding diffraction-limited widefield interpolations, and error maps. Furthermore, there will be a parameter sweep map of the FRC resolution estimate and the RSP fidelity metric of each eSRRF reconstruction.

The parameter “# frames for SRRF” defines the number of frames that will be used for every frame in the eSRRF reconstructed image. In the case of the Parameter Sweep, at least two frames are needed on the reconstructed image to perform FRC analysis. This example consists of a stack of 200 frames; therefore, 100 frames are chosen to do SRRF, which will yield a reconstructed image with 2 frames, each corresponding to the average of each subset of 100.

By clicking on “Show advanced settings”, further parameters can be selected.



2. To qualitatively compare the different reconstructions and spot severe artifacts, the grey scaling in the eSRRF stack can be reset by selecting the respective output image stack “[name of the file] (AVG)” (or “[name of the file] (VAR)”, etc., in case other reconstructions are chosen) and running the plugin function “*Fiji → Plugins → NanoJ-eSRRF → eSRRF – Tools → Reset stack for display...*”.
3. For a quantitative comparison of the reconstruction performance calculate the QnR map by running “*Fiji → Plugins → NanoJ-eSRRF → eSRRF – Get QnR...*” and select the RSP and FRC maps produced by the parameter sweep.



The QnR table is a matrix with the QnR values with the different chosen sensitivities in the rows (y values) and the radii in the columns (x values). The exact value can be inspected by positioning the cursor over a square and reading the values on the Fiji bar. The maximum QnR value and the corresponding parameter set are printed as an output in the “Log” window.

4. Choose the maximum value and visually inspect the corresponding image by scrolling in the stack “[name of the file] (AVG)” to check if it is acceptable.
5. To refine the optimal parameters, rerun the parameter sweep by choosing parameters around the optimal value and a reduced radius step size. In the case that the optimal is found in one of the borders of the QnR table, in the next sweep, larger or smaller parameters have to be chosen to make sure the optimum is found within the sweep area.

1.4 Reconstructing the image with eSRRF using the optimal parameters

Based on the optimal parameter set the full raw data can now be reconstructed.

1. Select the full raw data image stack and run “*Fiji* → *Plugins* → *NanoJ-eSRRF* → *eSRRF – Analysis*”

eSRRF v1.1.0

== eSRRF parameters ==

Magnification (default: 5)

Radius (pixels, default: 1.5)

Sensitivity (default: 1)

frames for SRRF (0 = auto)

☒ Vibration correction

== Reconstructions ==

☒ AVG reconstruction (default: on)

☐ VAR reconstruction (default: off)

☐ TAC2 reconstruction (default: off)

☐ Wide-field interpolation (default: off)

== Rolling analysis ==

☐ Perform rolling analysis (default: off)

frame gap between SR frame (0 = auto)

== 3D eSRRF ==

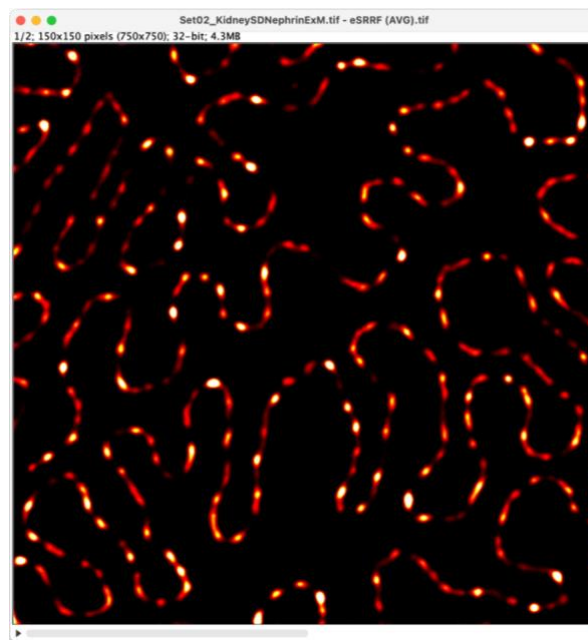
☐ Perform 3D-eSRRF from MFM data

Axial offset (in nm):

== Advanced settings ==

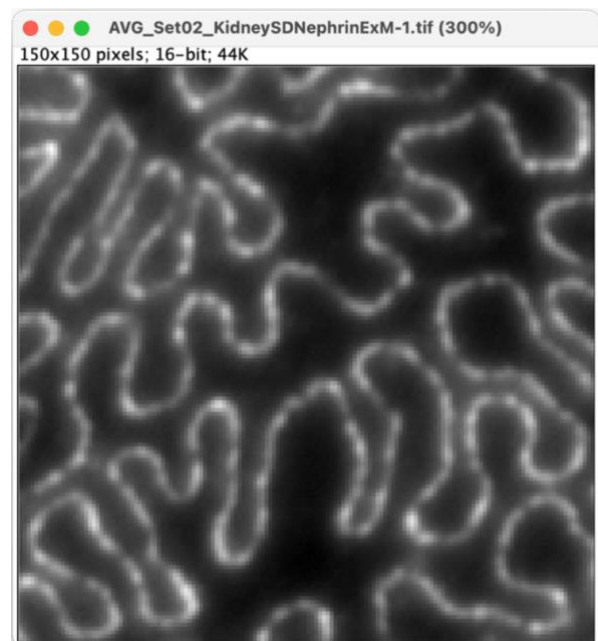
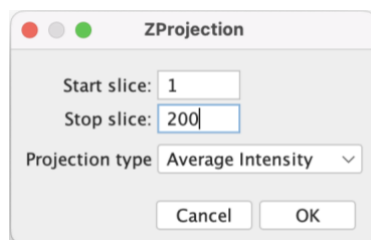
☐ Show advanced settings

Which results in the following eSRRF reconstructed image:

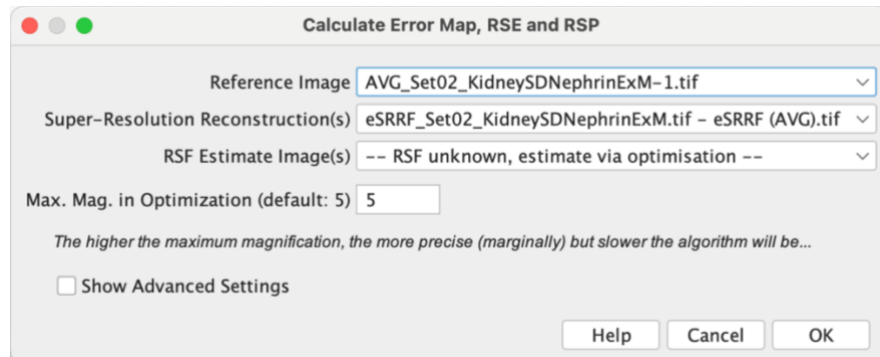


1.5 Assessing the reconstruction error with SQUIRREL

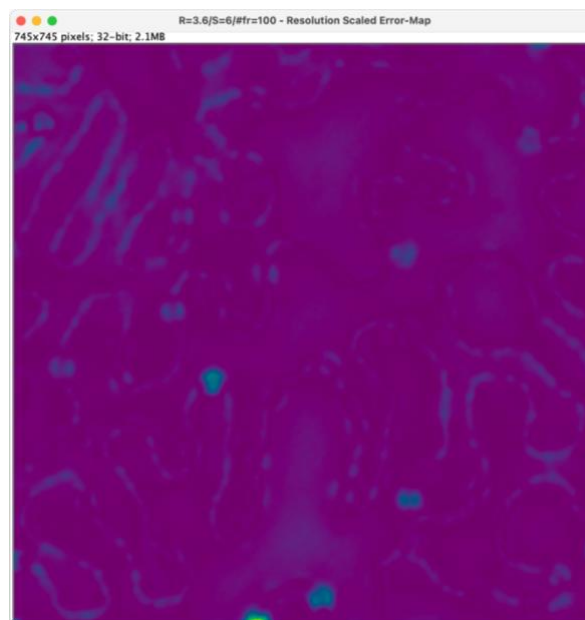
1. To find the error of the reconstruction, project the image in Z as an average (“Fiji → Image → stack → z-projection”, Projection type “Average Intensity”) to use it as diffraction-limited reference.



2. Calculating the error map: Run the error map plugin “*Fiji → Plugins → NanoJ-SQUIRREL → Calculate Error map*” selecting the average image as Reference Image and the eSRRF reconstructed image as Super-Resolution Reconstruction.



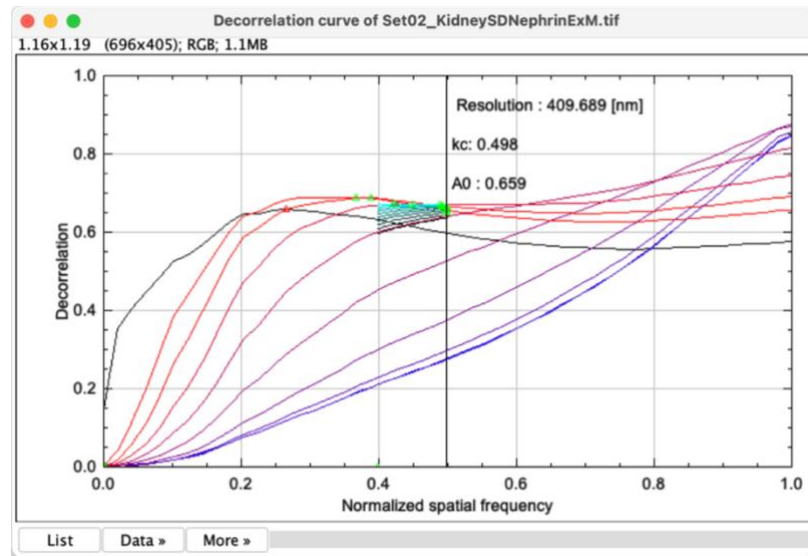
3. The plugin will output the global RSP and RSE values in a table and the local RSE distribution as an error map image.



1.6 Alternative resolution assessment approach with Decorrelation analysis

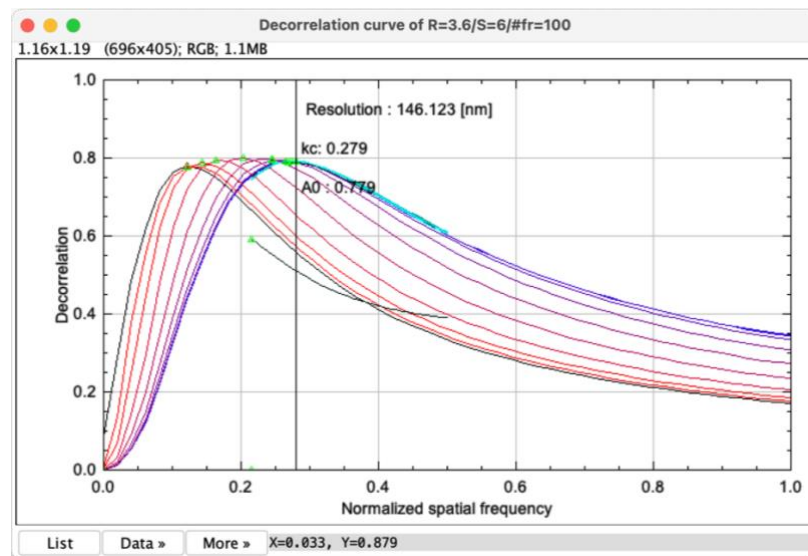
1. Installation: Download the plugin ImageDecorrelationAnalysis (ImageDecorrelationAnalysis_plugin.jar) from the GitHub repository (<https://github.com/Ades91/ImDecorr>). Copy the .jar-file ImageDecorrelationAnalysis_plugin.jar in your ImageJ plugins folder (.../ImageJ/plugins). After restarting ImageJ and the plugin will appear in the plugin list, under: “*Fiji → Plugins → ImageDecorrelationAnalysis*”
2. Average the diffraction limited image stack by Z-projecting it (*Fiji → Image → Stacks → Z project → projection type “Average intensity”*). This will be the diffraction-limited image that we want to characterize. Run the plugin (“*Fiji → Plugins → ImageDecorrelationAnalysis*”) with the default parameters. Make sure

that the pixel size of the image is the correct one (can be specified in “*Fiji* → *Image* → *Properties*”) before running, in this case is 102 nm. The plugin outputs the decorrelation analysis and the computed resolution.



Here the decorrelation analysis-based resolution estimate for the diffraction-limited reference is 410nm.

3. To check the resolution enhancement by the fluctuation-based super-resolution processing, repeat the process with the eSRRF reconstructed image.



Here the resolution estimate is 146 nm. Note that the correct pixel size has to be input in the image properties (in this case, with a magnification factor of 5 the pixel size of the reconstructed image is $102/5 = 20.4\text{nm}$).

Chapter 2: eSRRF Parameter optimisation in NanoPyx jupyter notebooks

2.1 Software installation

2.1.1 Installing python

The basic requirement for this pipeline is that Python be installed on the computer. One option is to install a package manager that will install Python for us. One of the best and fastest current package managers is Mamba: <https://mamba.readthedocs.io/en/latest/installation/mamba-installation.html>. Nevertheless, other ways to install Python can be used if desired.

To install Python from scratch, read the section "How to install Mamba" or follow the instructions at <https://github.com/conda-forge/miniforge>. Then, download the installer for Miniforge3 for your computer's specific architecture.

Once installed, the command prompt will display the word (base) in front of every prompt, indicating that the environment is, by default, the base environment, containing the basic libraries to work with Python and not meant for coding. Python and Mamba allow the creation of environments, which are isolated "coding" spaces that enable the installation of more advanced libraries. These environments enable you to start afresh without impacting fundamental Python components if issues arise during a specific installation.

To run the notebooks, run the cells individually by clicking the play button or the "shift+enter" keys. When a small GUI appears, interact with it as the notebook explains. Remember that the images are saved in the folder where the loaded file is.

The test datasets can be downloaded here: <https://zenodo.org/doi/10.5281/zenodo.11518140>.

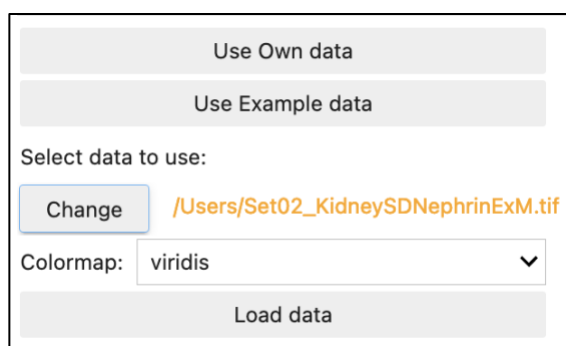
2.1.2 Installing Nanopyx library

1. Create a new python environment and install NanoPyx as explained in <https://github.com/HenriquesLab/NanoPyx>
2. In short:
 - a. Open the console/terminal and create a new conda environment: "conda create --name localize_render python=3.10"
 - b. Activate the environment: "conda activate nanopyx"
 - c. Install NanoPyx package using: "pip install nanopyx[jupyter]"
3. Download the notebooks of interest from <https://github.com/HenriquesLab/NanoPyx/tree/main/notebooks>
4. Run the notebook by writing "jupyter lab" and navigating to the notebook folder

Note that the output reconstructions and figures computed in the following notebooks will be stored in the folder of the image being reconstructed.

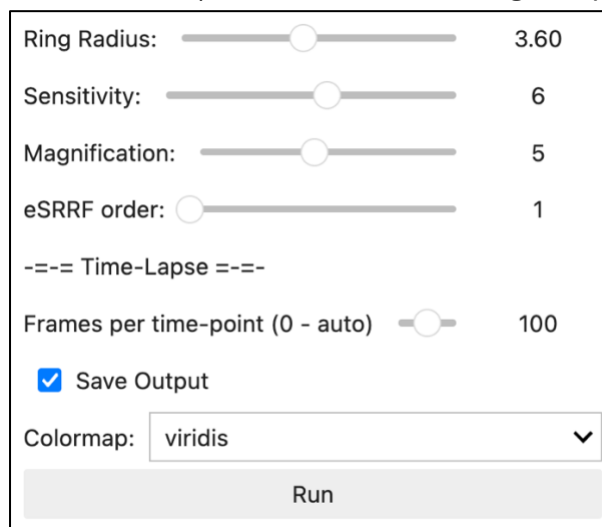
2.2 eSRRF processing with NanoPyx-eSRRF

1. Run the notebook for eSRRF processing and image quality control: <https://github.com/HenriquesLab/NanoPyx/blob/main/notebooks/eSRRFandQC.ipynb>
2. Run the **cells 1-3** of the notebook. This will import the necessary libraries and connect to Google Drive to use example data in the notebook.
3. To use the desired image, on the buttons after cell 3, select “Use Own data” and press “Select”. This will enable a window to find and select the image to upload. Once selected, press again “Select” and click “Load data”.



The screenshot shows a user interface for selecting data. At the top are two buttons: "Use Own data" and "Use Example data". Below them is the text "Select data to use:". There is a "Change" button and a text field containing the file path "/Users/Set02_KidneySDNephtrinExM.tif". Below the text field is a "Colormap:" label and a dropdown menu currently set to "viridis". At the bottom is a "Load data" button.

4. Run **cell 4** and select the desired values. You can select the values with the scrollbars and in the numbers (in that case, do not forget to press intro)



The screenshot shows a parameter adjustment interface. It includes several sliders with corresponding numerical values: "Ring Radius" (3.60), "Sensitivity" (6), "Magnification" (5), and "eSRRF order" (1). Below these is a section labeled "-- Time-Lapse --" containing a slider for "Frames per time-point (0 - auto)" set to 100. There is a checked checkbox for "Save Output". At the bottom, there is a "Colormap:" label with a dropdown menu set to "viridis" and a "Run" button.

Press Run to produce the eSRRF image. The image is saved on the folder of the imported raw data.

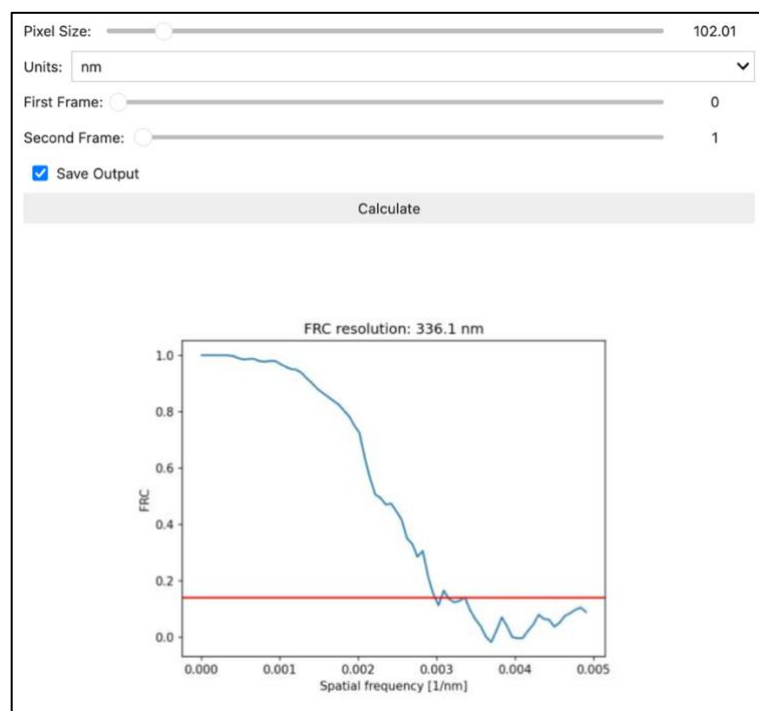
2.3 Calculate errors in the eSRRF processed image.

NanoPyx library has Error Map, FRC, and Decorrelation analysis implemented. The **cells 5-10** of the notebooks are dedicated to the Error Map of the reconstruction and the FRC and Decorrelation analysis for both the diffraction-limited and the super-resolved image.

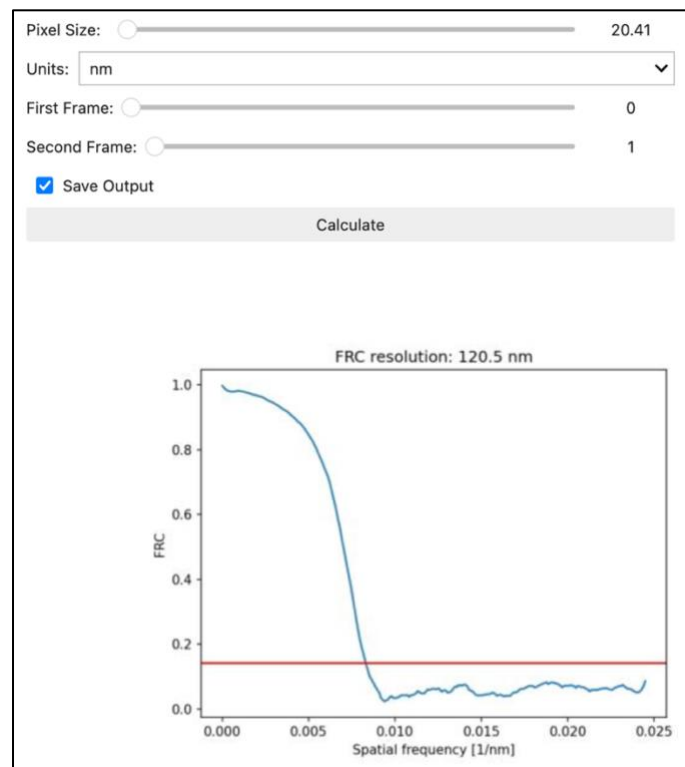
1. To get the Error Map, after running the previous cells and computing the SRRF images, run the **cell 5**.



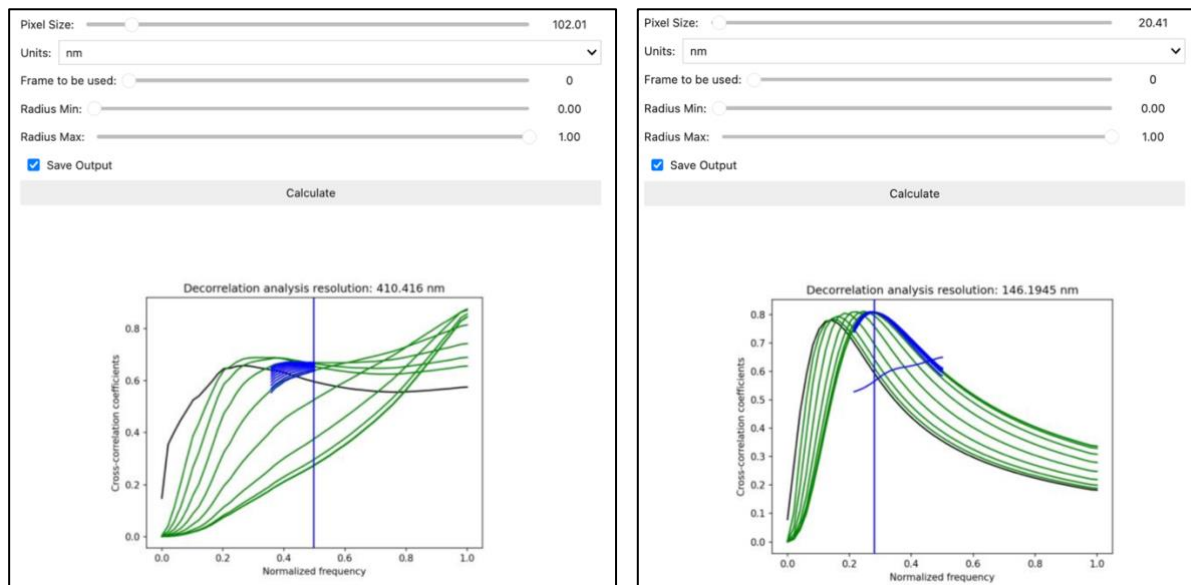
2. To calculate the FRC of the diffraction-limited image, run **cell 6**. Then, press "Calculate" after choosing the correct pixel size of the image and the desired frames to use for FRC computation.



3. Run **cell 7** to calculate FRC on the reconstructed image with the correct pixel size.



4. Run **cell 8** to calculate decorrelation on the diffraction limited image (left) and the super-resolved reconstruction (right) with the correct pixel size for each.



This will result in a resolution estimate of 410 nm for the diffraction limited and 146 nm for the super-resolved image.

Chapter 3: eSRRF reconstruction in example images

3.1 Test datasets provided

Three test datasets are provided to showcase the eSRRF reconstruction process:

1	Set01_DNA-PAINT_Microtubules.tif
2	Set02_KidneySDNephrinExM.tif
3	Set03_simulation_groundTruth_2p5Sigma - Fluorescence stack_Avg5.tif

They are available for download here: <https://zenodo.org/doi/10.5281/zenodo.11518140>

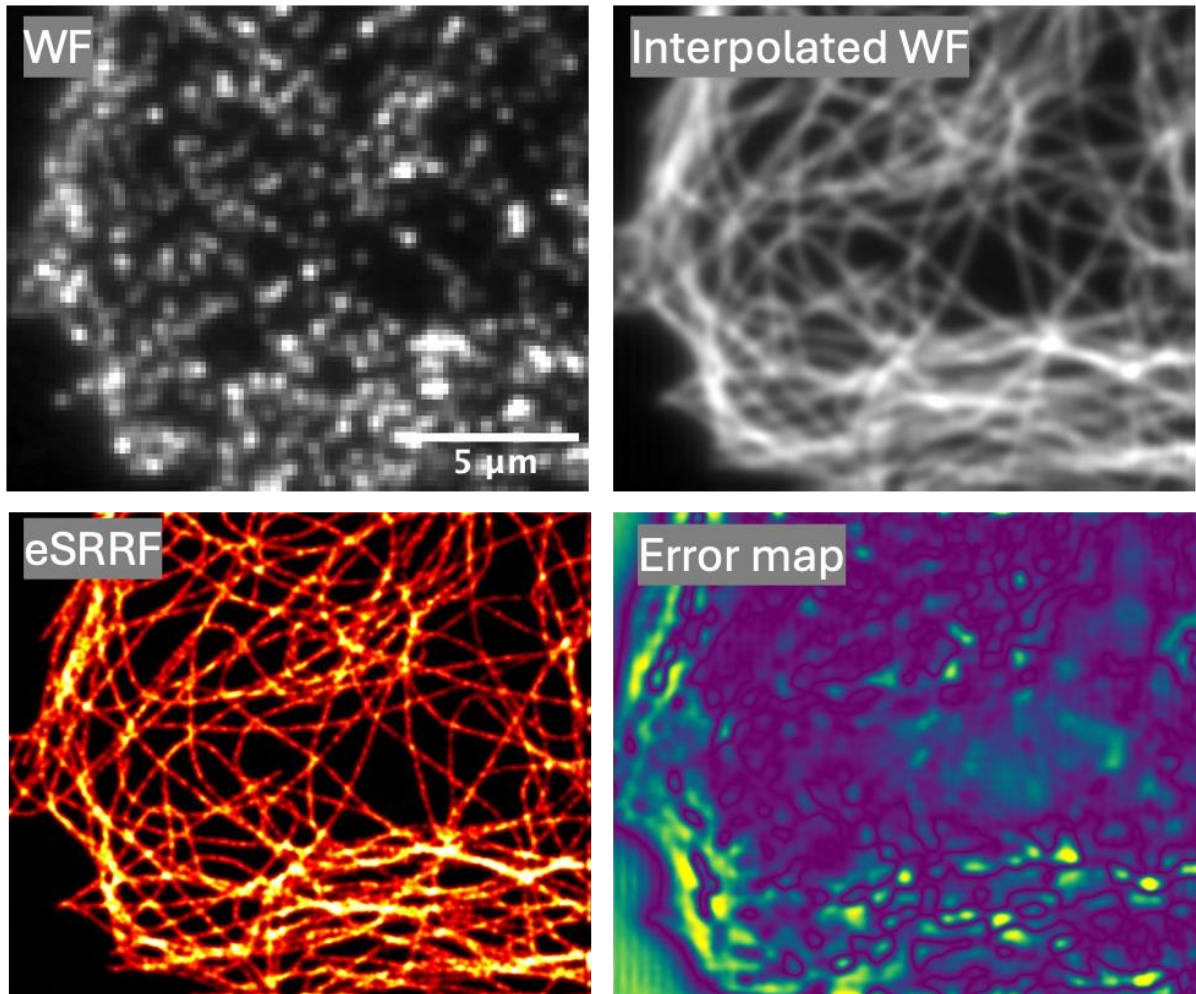
- 1) Microtubules sample.** DNA-PAINT microscopy measurement of immunolabeled microtubules in fixed COS-7 cells, showing 0.121 localizations per frame and μm^2 (data published in [Laine and Heil et al.](#))
- 2) Kidney sample.** Human kidney biopsies stained with nephrin (data published in [Kylies et al.](#))
- 3) Emitters simulation.** Simulated individual molecules emitting placed on concentric rings with radii increasing by 220 nm steps. On each ring, the molecules are separated by 57.5, 115, 173, 230, 288, and 345 nm, respectively (data published in [Laine and Heil et al.](#))

3.2 Optimal values for reconstruction

One set of optimal parameters is provided below. The parameters were found by running the parameter sweep, as explained in Chapter 1.

Sample	Pixel size	Type of reconstruction	Magnification	Radius	Sensitivity	Vibration corrected
1	160nm	VAR	5	1.7	1	No
2	102nm	AVG	5	3.6	6	Yes
3	100nm	VAR	5	0.75	4	No

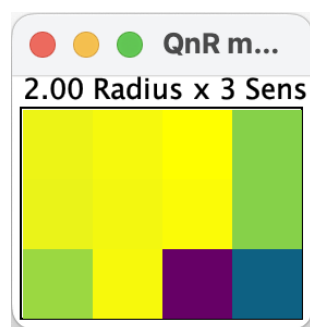
3.2.1 Microtubules sample



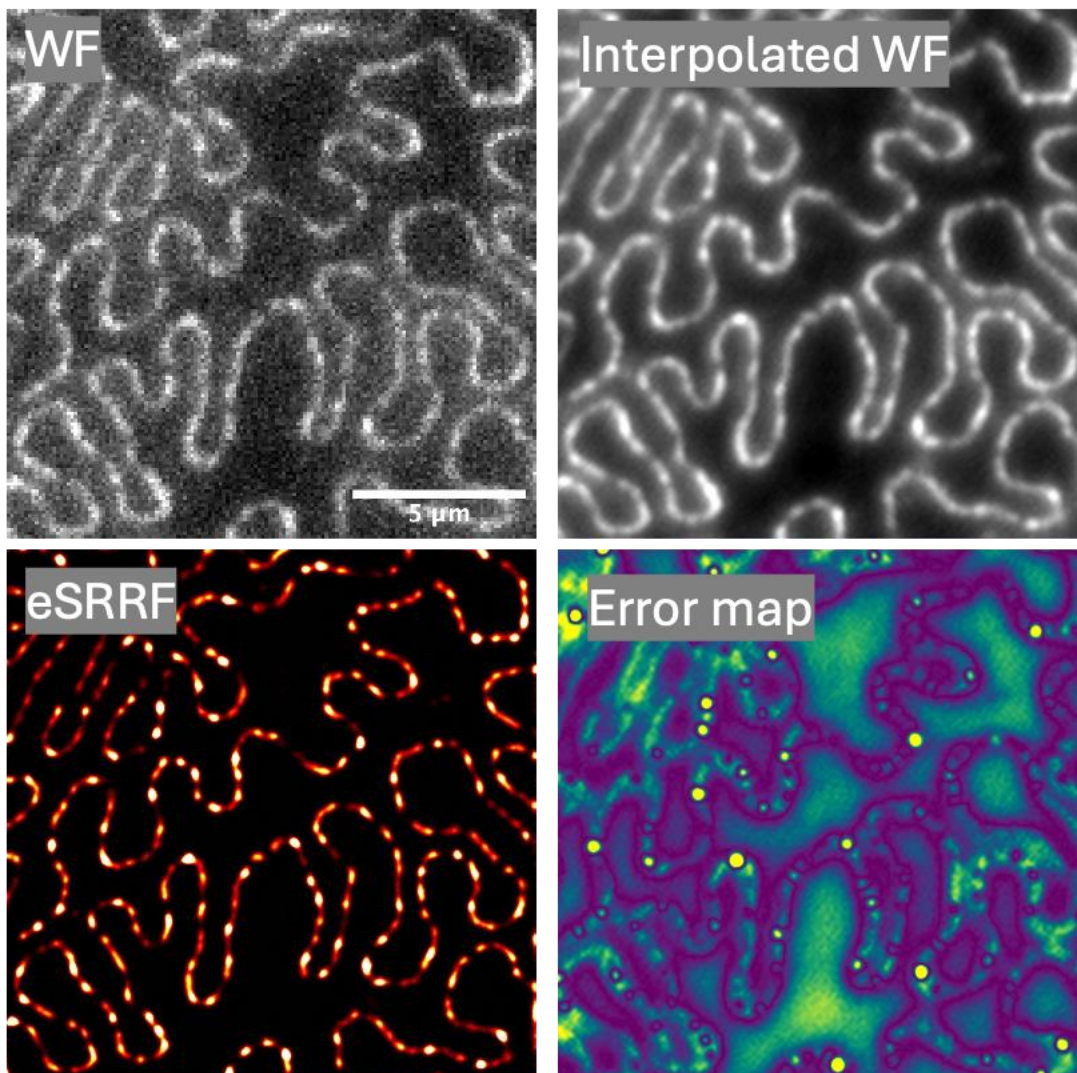
To find the optimal parameters, the parameter sweep plugin is run with the following parameters (type or reconstruction: VAR):

- Radius: Start 0.7, Delta 0.50, Number 4
- Sensitivity: Start 1, Delta 1, Number 3
- # frames for SRRF: Start 250, Delta 1, Number 1
- Vibration correction off

The resulting QnR map is as follows map presents a maximum for **R = 1.7** and **S = 1**:



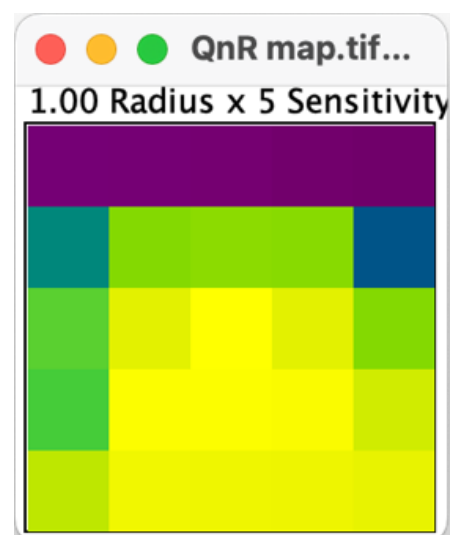
3.2.2 Kidney sample



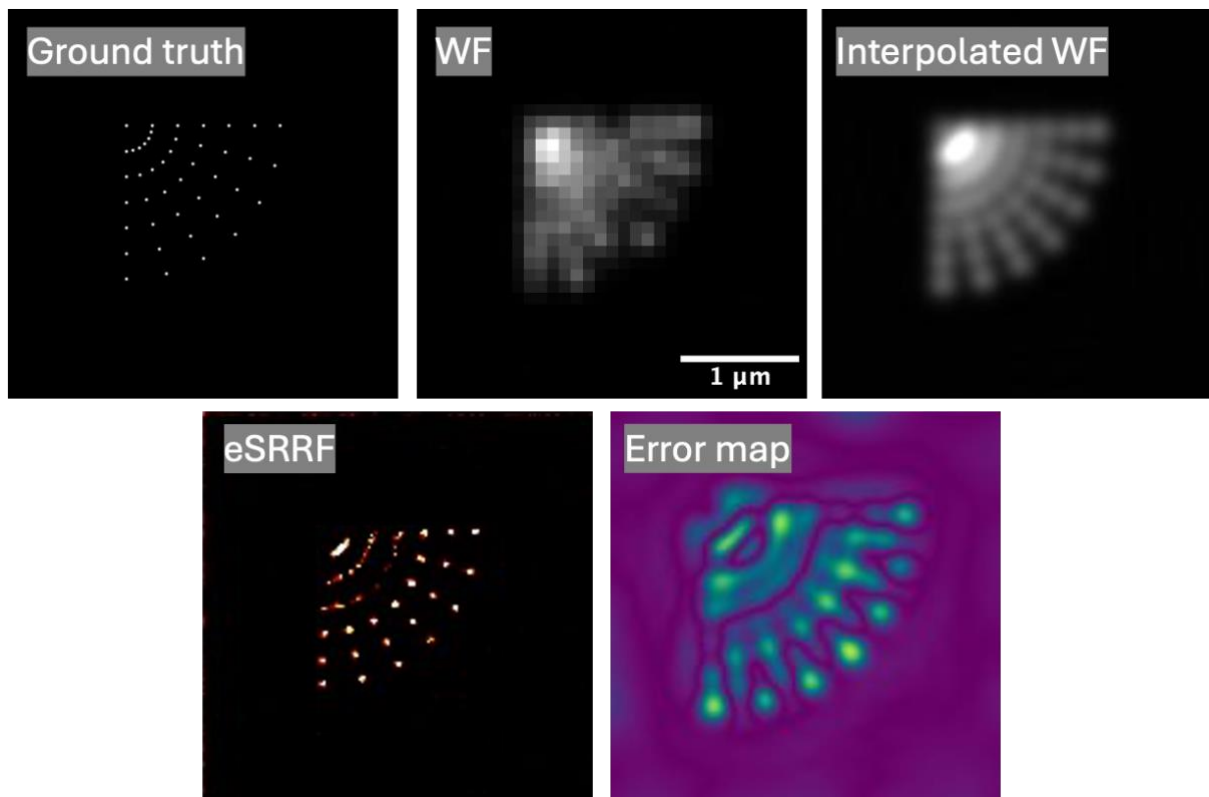
To find the optimal parameters, the parameter sweep plugin is run with the following parameters (type or reconstruction: AVG):

- Radius: Start 3.2, Delta 0.20, Number 5
- Sensitivity: Start 1, Delta 1, Number 5
- # frames for SRRF: Start 100, Delta 1, Number 1
- Vibration correction active

The resulting QnR map is as follows map presents a maximum for **R = 3.6** and **S = 6**:



3.2.3 Emitter simulation



To find the optimal parameters, the parameter sweep plugin is run with the following parameters (type or reconstruction: VAR):

- Radius: Start 0.25, Delta 0.25, Number 8
- Sensitivity: Start 3, Delta 1, Number 3
- # frames for SRRF: Start 50, Delta 1, Number 1
- Vibration correction off

The resulting QnR map presents a maximum for **R = 0.75** and **S = 4**:

