

# A napari FF-SRM plugin for advanced microscopy image processing.

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## **USER MANUAL**

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A napari FF-SRM plugin for advanced microscopy image processing.

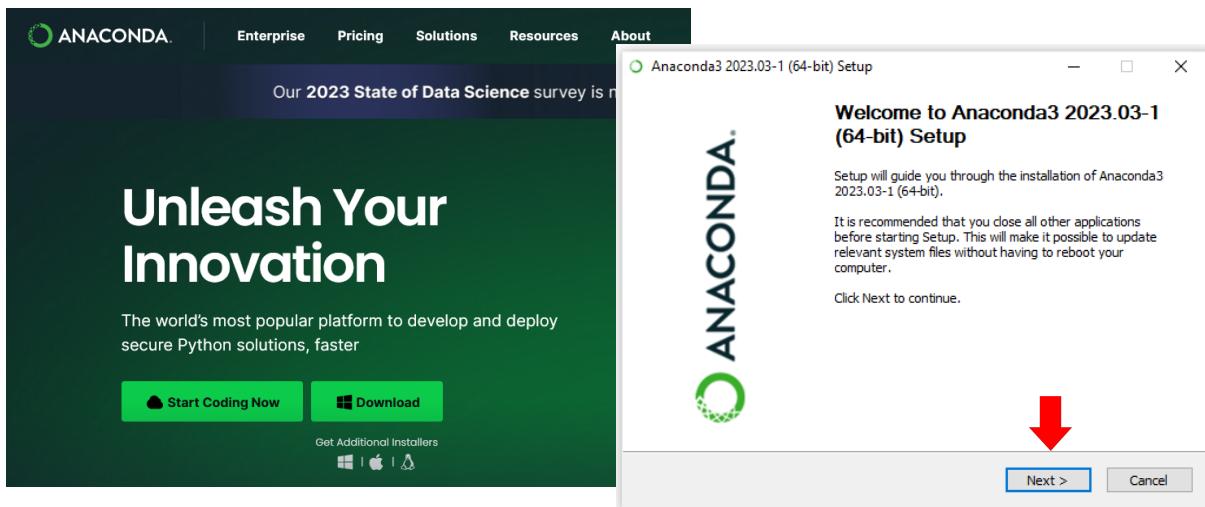
## 1. Installation process.

Review the system requirements before installing Anaconda Distribution. Select the installers for your computer's operating system:

<https://docs.anaconda.com/free/anaconda/install/index.html>

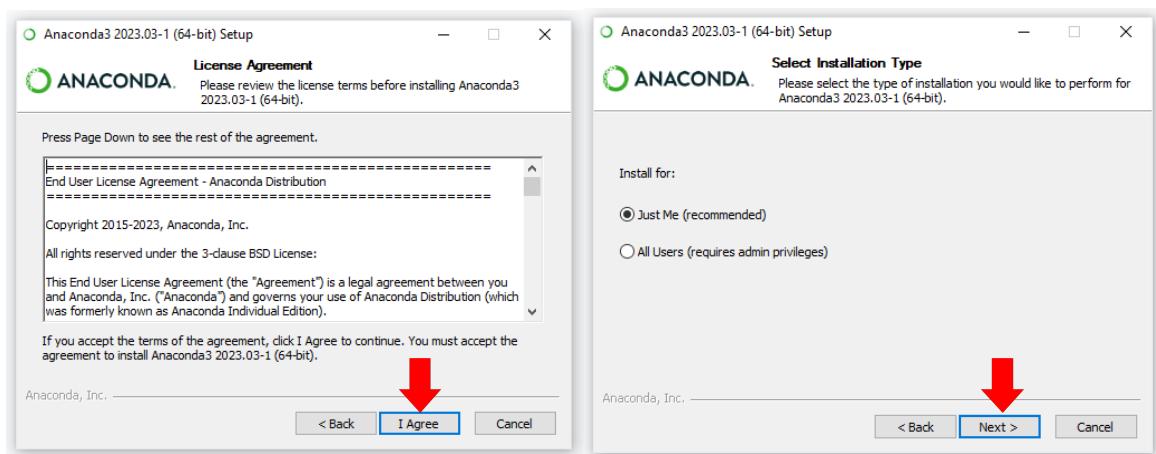
### 1.1 Installing Anaconda on Windows.

- 1) Download the Anaconda installer from <https://www.anaconda.com/> (fig. 1, left).
- 2) Go to the "Downloads" folder and double-click the **installer** to launch.
- 3) Click "**Next**" on the Setup window (fig. 1, right):



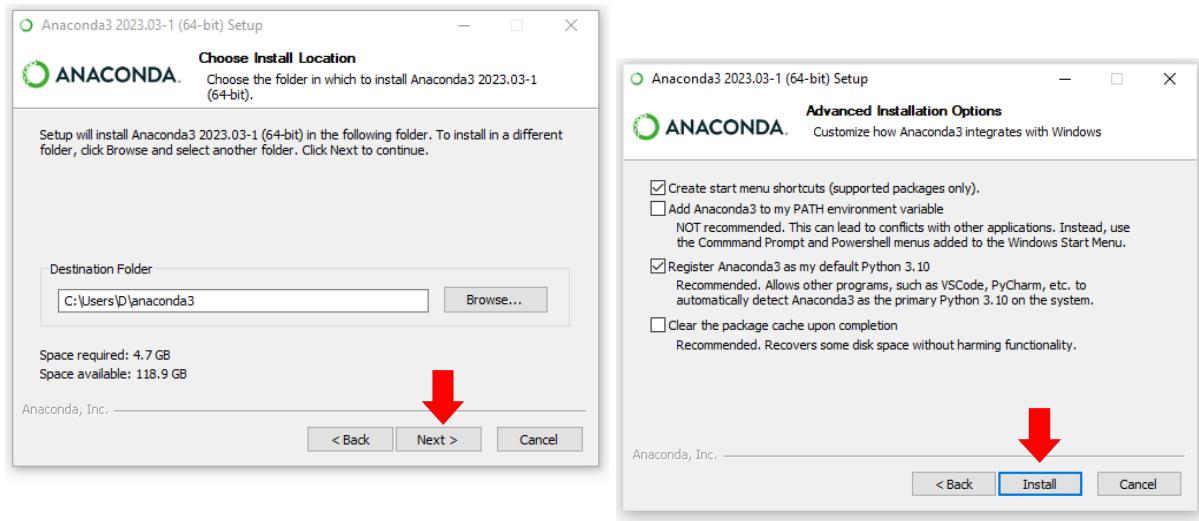
**Figure 1.** Installation windows for Anaconda Distribution. *Left:* Anaconda webpage. *Right:* Anaconda's setup window.

- 4) Read the licensing terms and click "**I Agree**" to continue (fig. 2, left).
- 5) It is recommended to install the program using the "**Just Me**" option, which will be limited to the current user account. However, if you wish the program available for all accounts on the computer, select "**All Users**". Please note that selecting the latter option will require Windows Administrator permissions. Click "**Next**" (fig. 2, right):



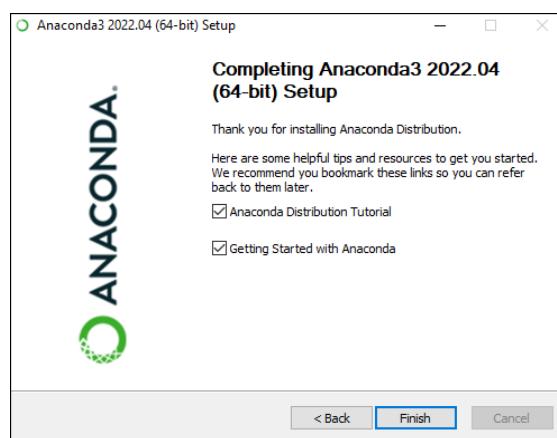
**Figure 2.** Anaconda installation Setup. *Left:* License agreement. *Right:* Anaconda's installation type.

- 6) Select an installation **location** (Destination Folder) and click “**Next**” (fig. 3, left).
- 7) Choose where to add Anaconda. It is recommended to register Anaconda software as your default Python because adding it to your PATH environment can interfere with other software. Click “**Install**” (fig. 3, right):



**Figure 3.** Anaconda installation Setup. *Left:* Installation location. *Right:* Anaconda's advanced installation options.

- 8) If you want to watch the packages Anaconda is installing, click “**Show details**”. Click “**Next**”.
- 9) After a successful installation you will see the “**Thanks for installing Anaconda**” dialog box.
- 10) Click the “**Finish**” button and verify the installation.



**Figure 4.** Anaconda installation Setup. *Left:* Installation details.

## 1.2 Installing Napari.

Napari is a fast, interactive and multi-dimensional image viewer for Python. To select which distribution to install and further details visit: <https://napari.org/stable/>.

For those familiar with Python, Napari can be installed on most macOS, Linux, and Windows systems using pip:

- 1) First install napari viewer: It is highly recommended to install napari into a clean virtual environment using an environment manager like [conda](#) or [venv](#). For example, with [conda](#):

```
conda create -y -n napari-env -c conda-forge python=3.9  
conda activate napari-env  
pip install "napari[all]"
```

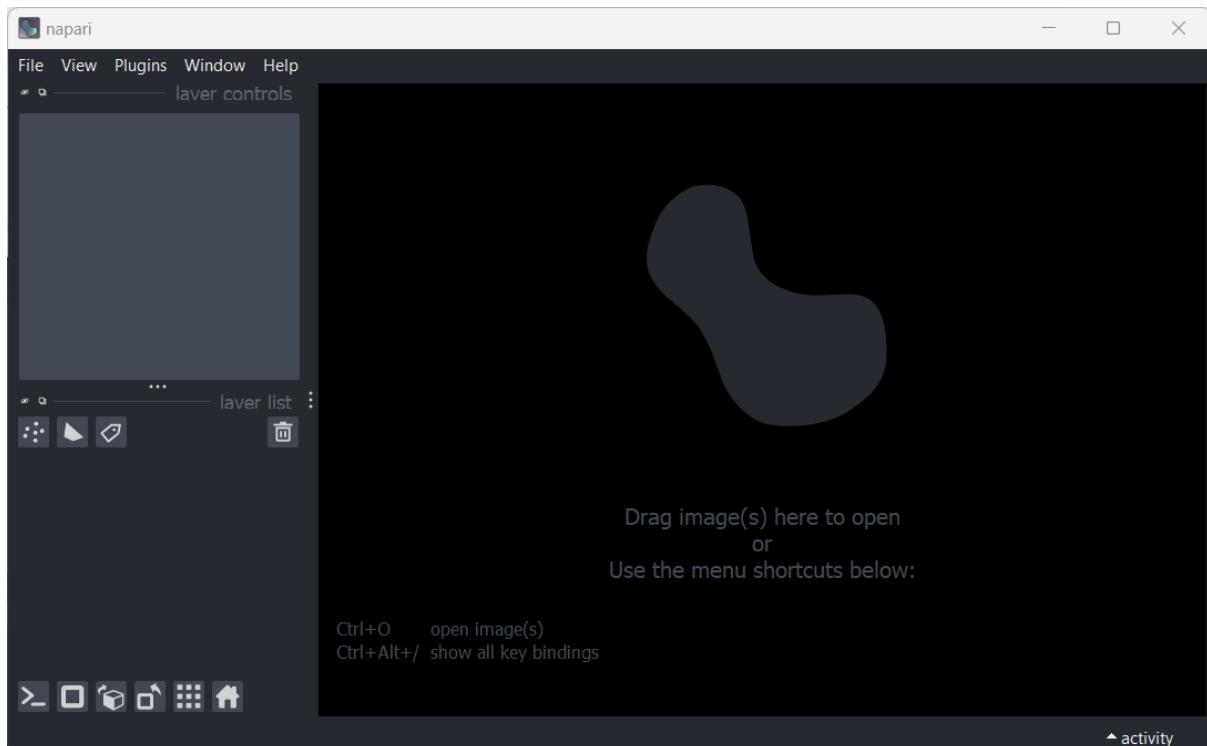
- 2) Open Napari from command line:

Once installed, simply run  
napari

For further information visit: [https://napari.org/stable/tutorials/fundamentals/quick\\_start.html](https://napari.org/stable/tutorials/fundamentals/quick_start.html)

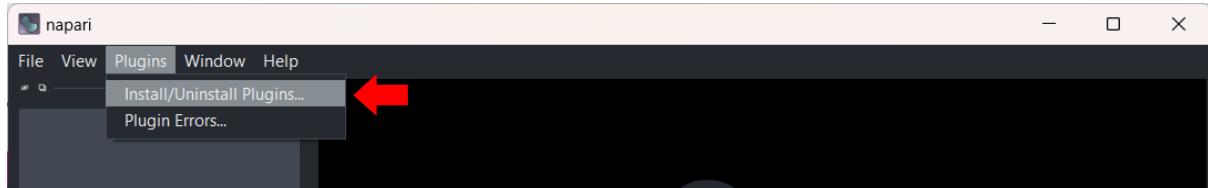
## 1.3 Installing napari-superres plugin.

- 1) When starting Napari, it will display the following window on the screen:



**Figure 5.** Napari's software window.

- 2) Select the “Plugins” Menu. Click the “Install/Uninstall Plugins” option (fig. 6).

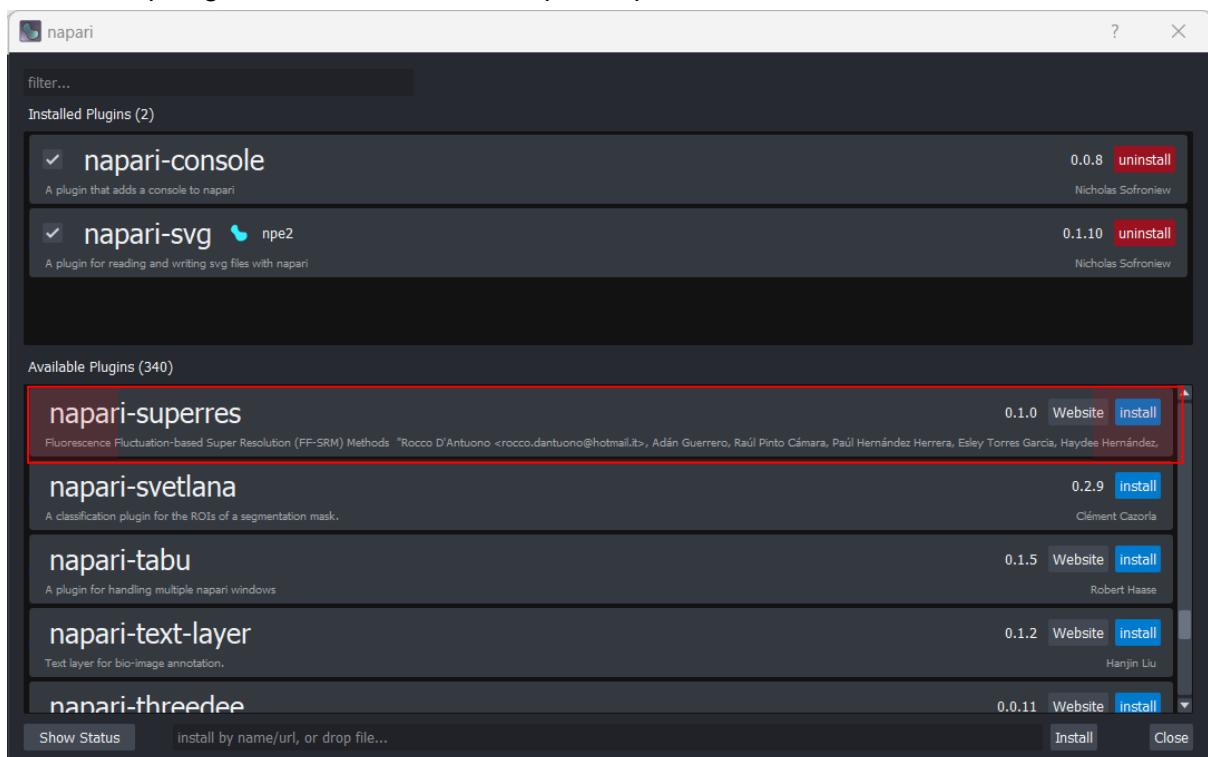


**Figure 6.** Napari installation plugin menu.

**3)** Install napari-superres.

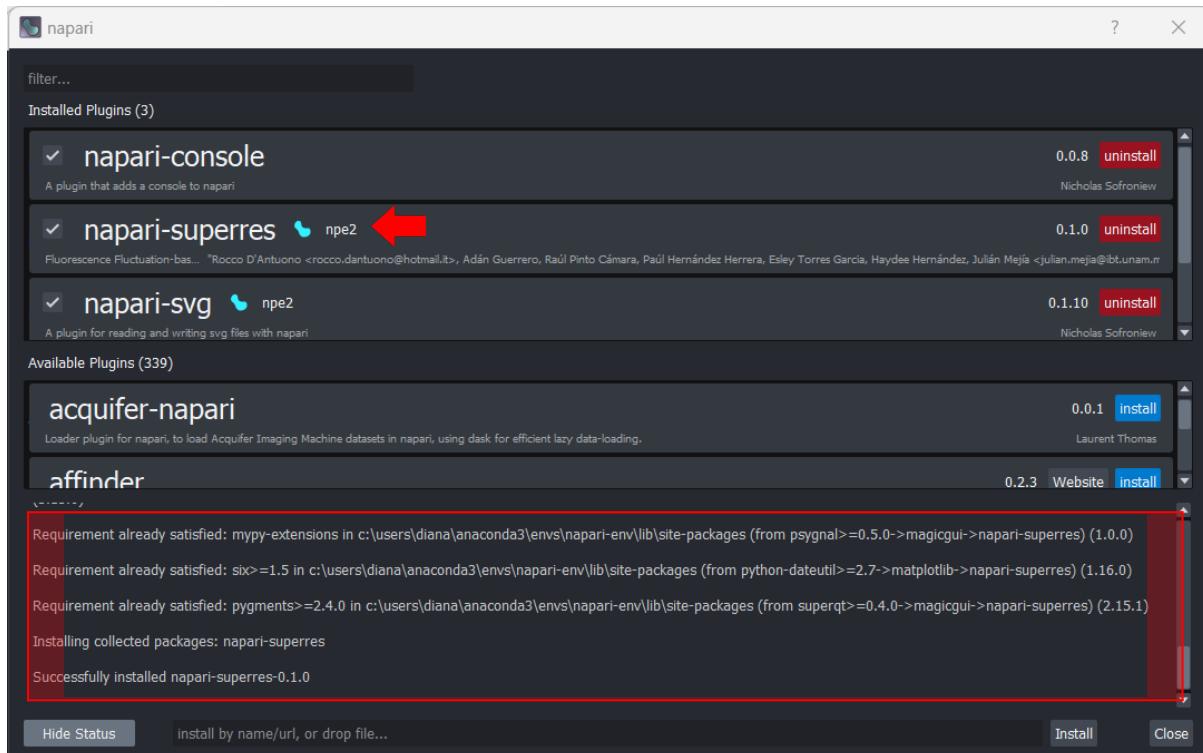
Simply search for the "napari-superres" plugin in the plugin install package interface and click install (fig. 7).

GitHub that contains the beta version of MSSR:  
<https://github.com/RoccoDAnt/napari-superres>



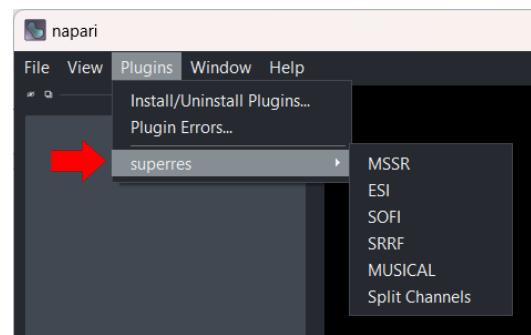
**Figure 7.** Search for the "napari-superres" (red rectangle) in the **Available Plugins** Napari menu to install.

**4)** Click "**Install**" and monitor the installation by clicking on **Show Status** option (fig. 8):



**Figure 8.** Napari-superres plugin installation process on Napari indicated by the red rectangle.

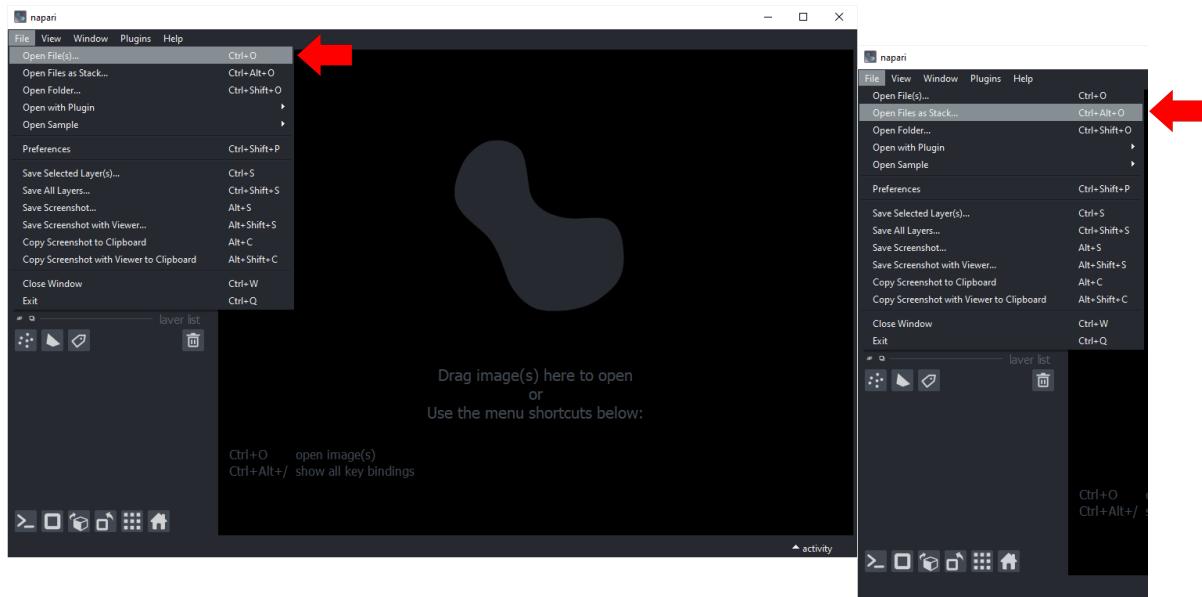
- 5) Once the plugin has been successfully installed, restart Napari and on the “Plugins” menu it will appear the napari-superres option and the following menu:



**Figure 9.** Napari-superres plugin installed on Napari.

## 2. Open Images in Napari.

- 1) Once you open the software, select the “File” menu (fig. 10, left) and you can select **Open File(s)** to browse directly from your computer or you can also open a **stack** of images (fig. 10, right).



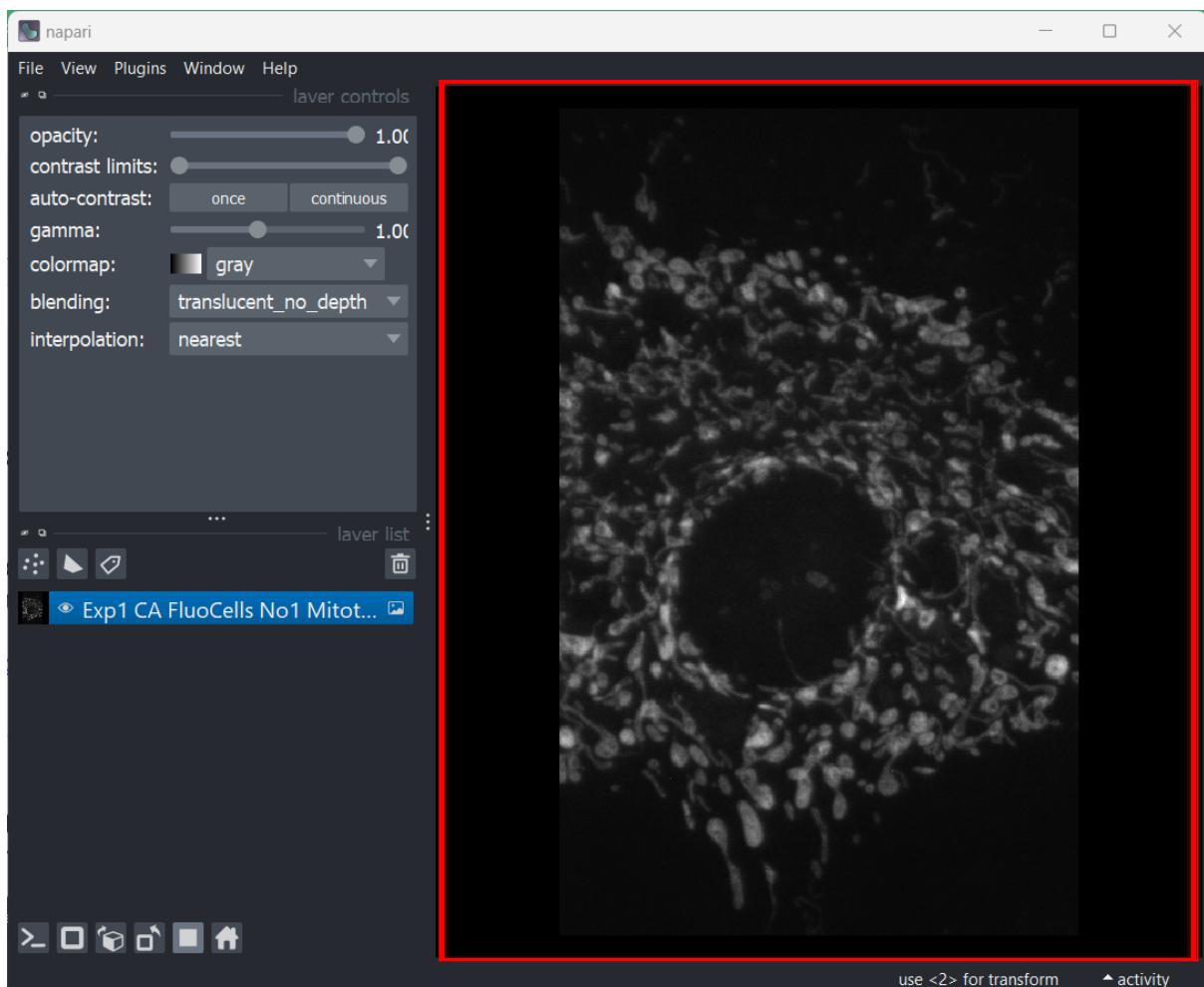
**Figure 10.** Napari software. *Left:* File menu, open file(s) option. *Right:* Open a stack of images.

## 2.2 Image preferences.

Currently, the Napari plugin has limited compatibility and is exclusively designed to open image files in the TIFF format.

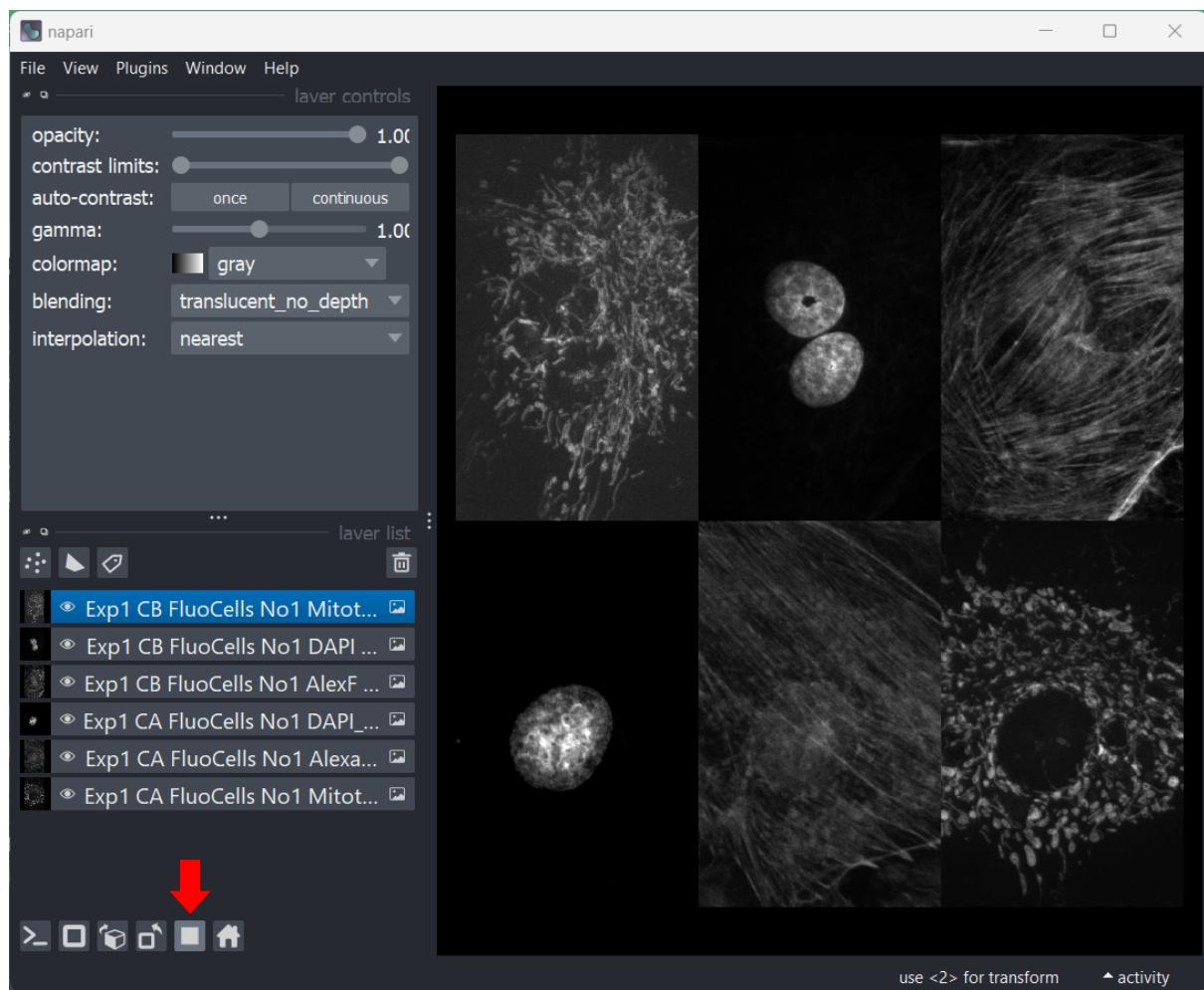
## 2.3 Multiple images visualization panel.

- 1) When you open a single image in Napari, it will provide a visualization that covers the entire right panel (indicated by a red rectangle, fig. 11):



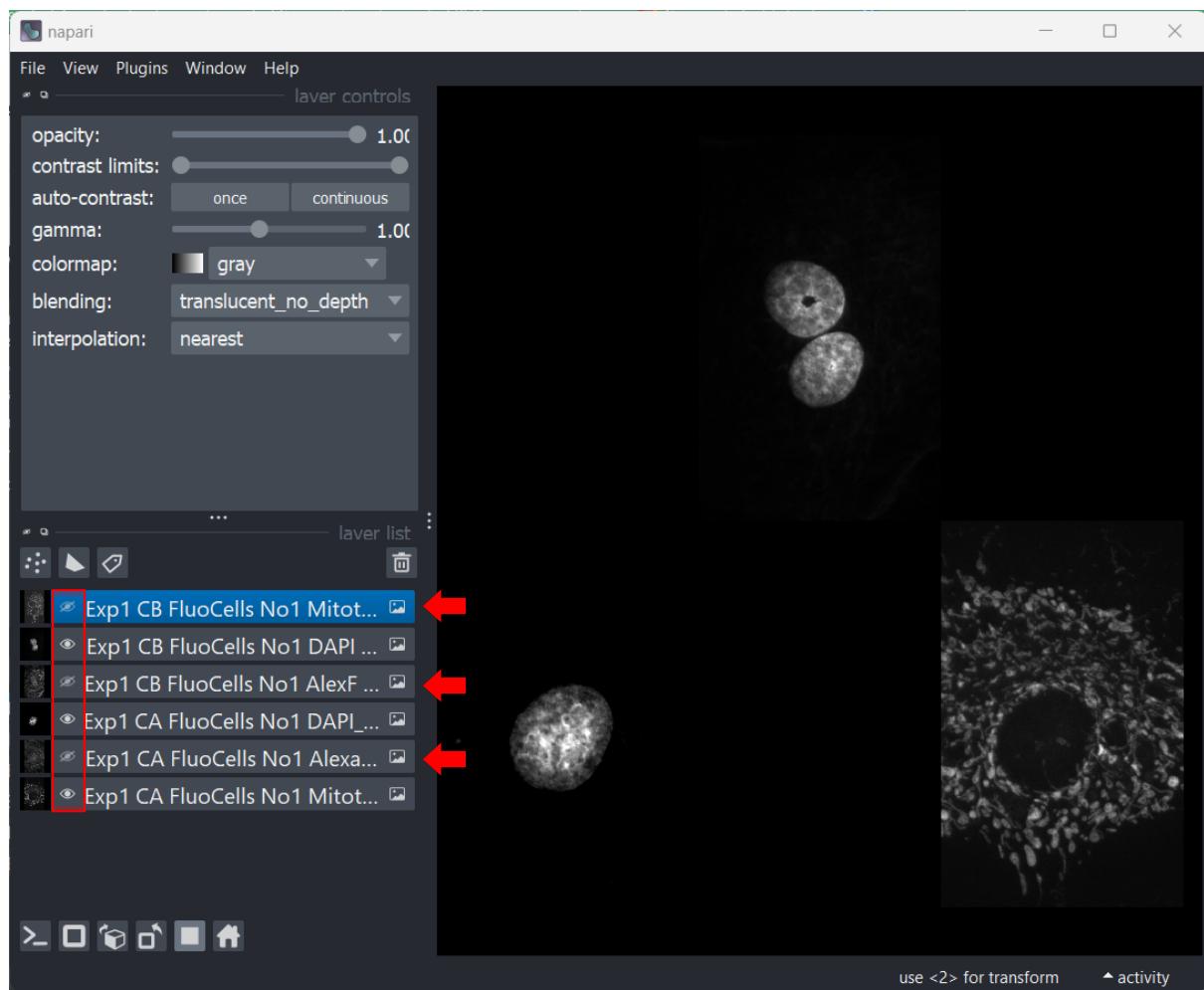
**Figure 11.** Napari visualization panel for a single image.

- 2) However, it can be highly beneficial to visualize multiple images on a single panel in order to compare them or differentiate between different dyes. To achieve this, you simply need to select the "**Toggle Grid Mode**" button (indicated by the red arrow in fig. 12) after opening all the desired images.



**Figure 12.** Napari visualization panel for multiple images: toggle grid mode (red arrow).

- 3) Once you are in this visualization mode, you have the option to hide one or multiple opened images. Simply click on the eye icon (indicated by a red rectangle) located to the left of each image file (fig. 13):



**Figure 13.** In the Napari visualization panel for multiple images, any hidden images are not displayed. You can identify these hidden images by the eye icon, which is crossed out with a slash symbol and indicated by a red arrow.

### 3. The Mean-Shift Super Resolution (MSSR) plugin tutorial.

#### 3.1 Selection of MSSR process.

- 1) Once you select the dataset from your computer's file system, Napari will display a visualization.

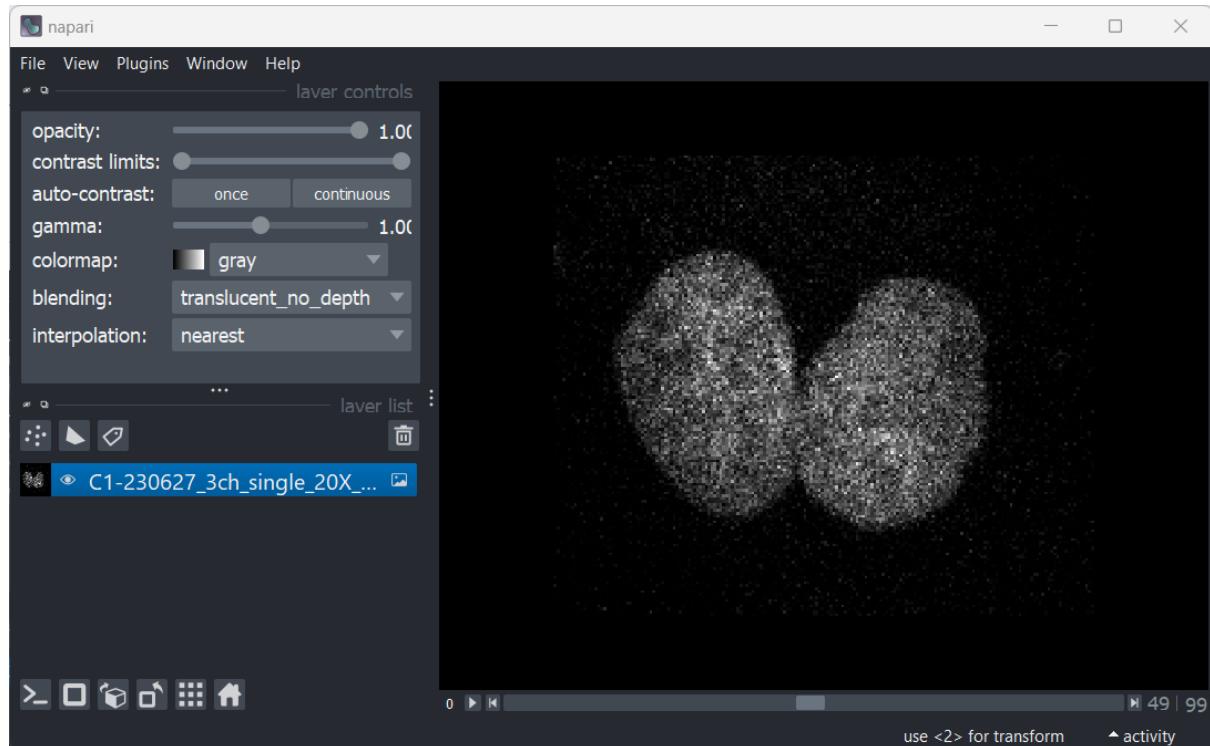


Figure 14. Napari's dataset visualization.

- 2) To use MSSR, select the "Plugins" menu and click on the "superres" option.

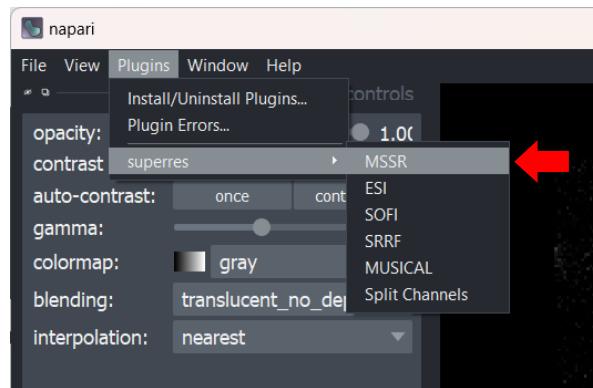
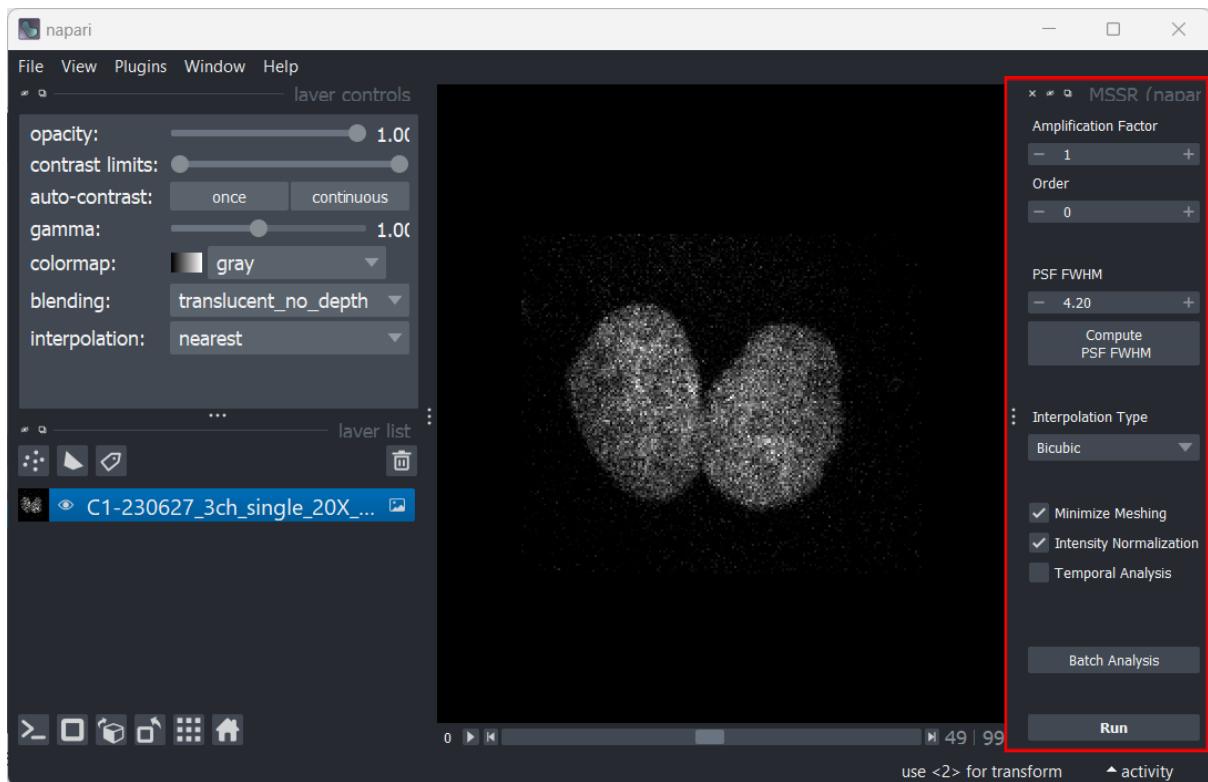


Figure 15. MSSR plugin.

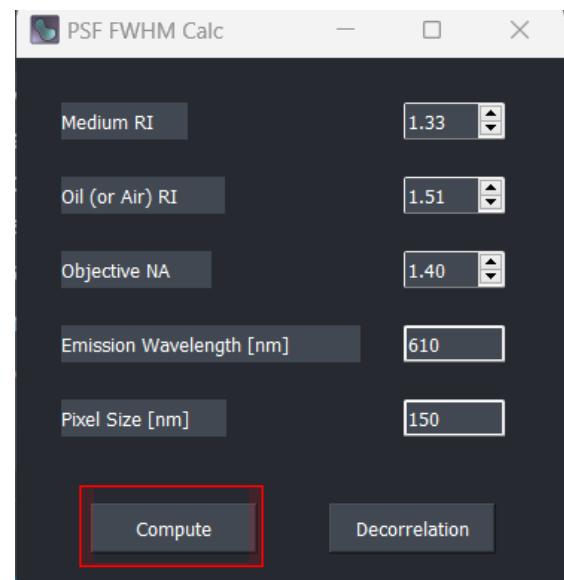
- 3) Once the MSSR plugin is selected, it will appear in the right section of the Napari window, providing the necessary parameters for applying MSSR analysis (fig. 16).



**Figure 16.** MSSR parameters menu (red rectangle).

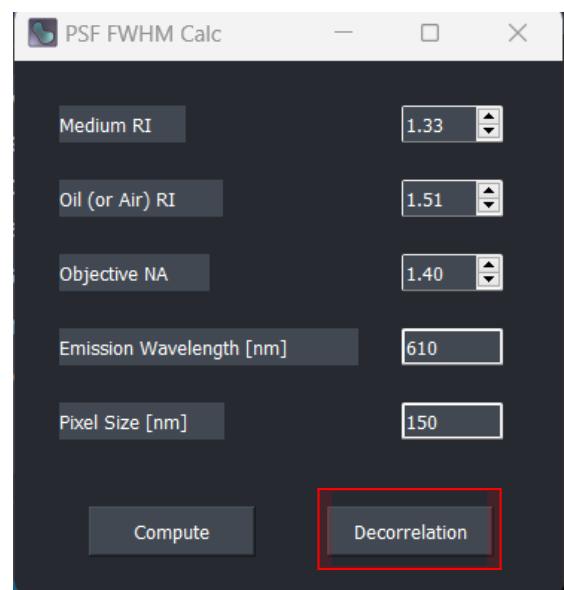
### 3.2 PSF FWHM estimation.

- 4) Fill in the required parameters with the necessary information and click "Compute" to obtain the ideal estimated value of the PSF FWHM using the calculator option.



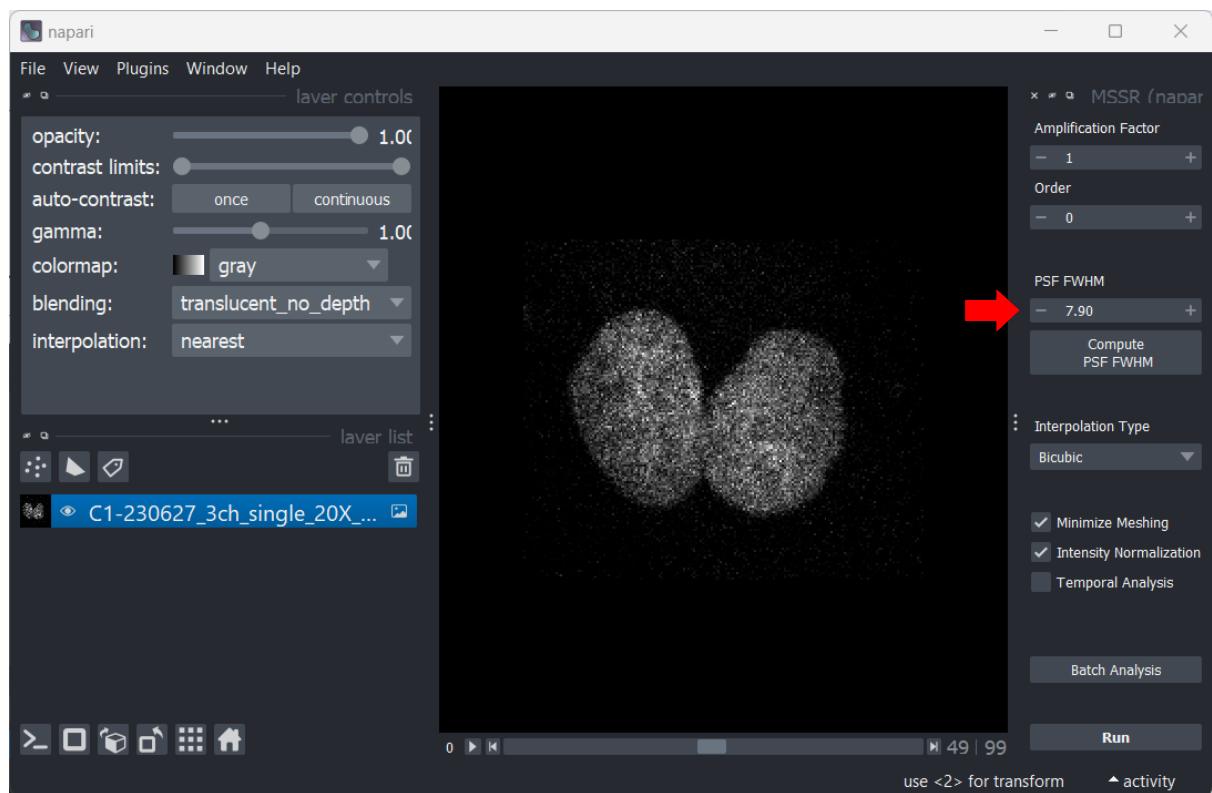
**Figure 17.** PSF FWHM estimation using the MSSR calculator option.

- 5) Select the "**Decorrelation**" option to estimate the PSF FWHM directly from the image of interest.



**Figure 18.** PSF FWHM estimation using the Decorrelation option.

- 6) The PSF FWHM value will be adjusted automatically:



**Figure 19.** PSF FWHM estimation using the Decorrelation option.

### 3.3 Single-frame MSSR analysis.

- 7) To perform **single-frame MSSR** analysis, click on "Run," and Napari will process the image and provide the resulting image (highlighted in blue text above the original data, fig. 20).

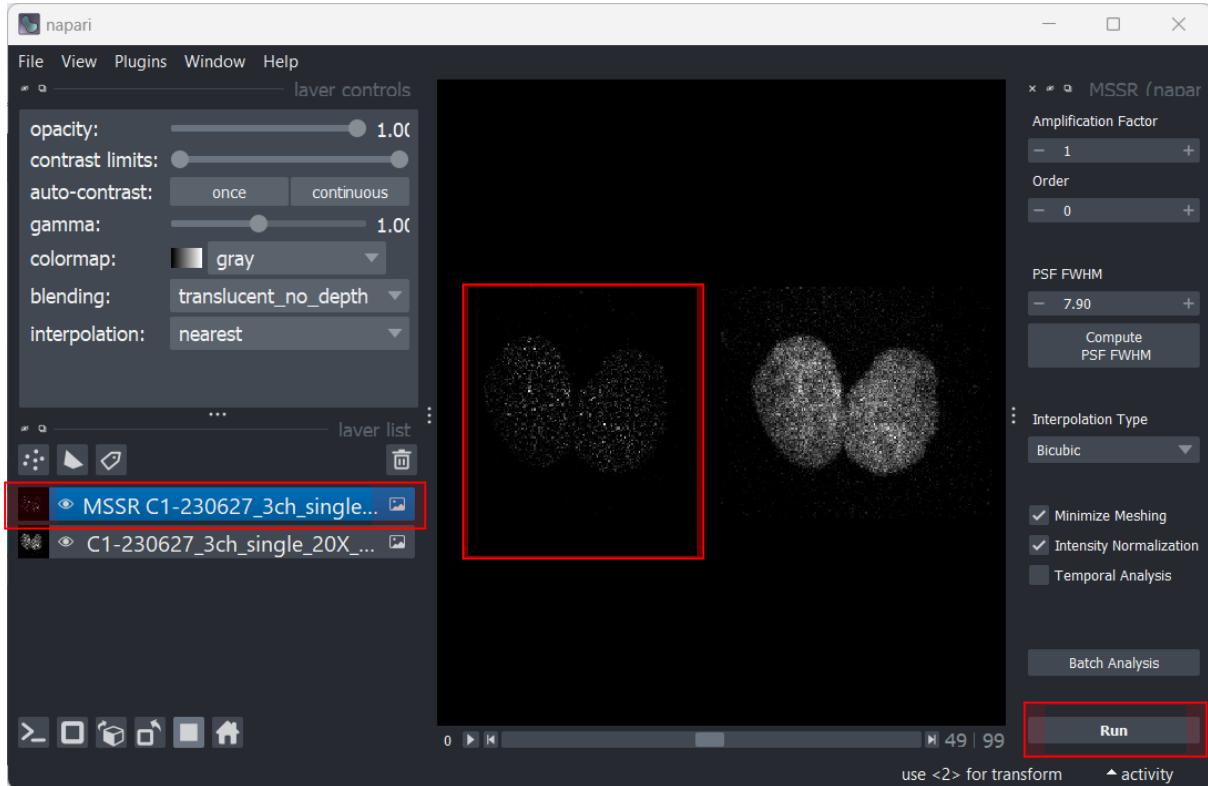


Figure 20. Single-frame MSSR analysis result.

### 3.3 Temporal MSSR analysis.

- 8) For **temporal MSSR** analysis, it is recommended to first change to the Fourier Interpolation type and then select the Temporal Analysis option. Napari will display the PTF menu, where you can choose the statistical integration method:

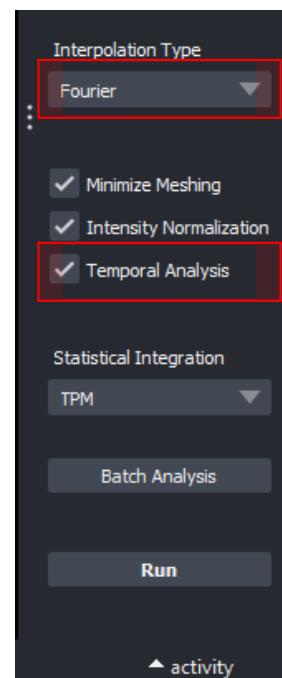
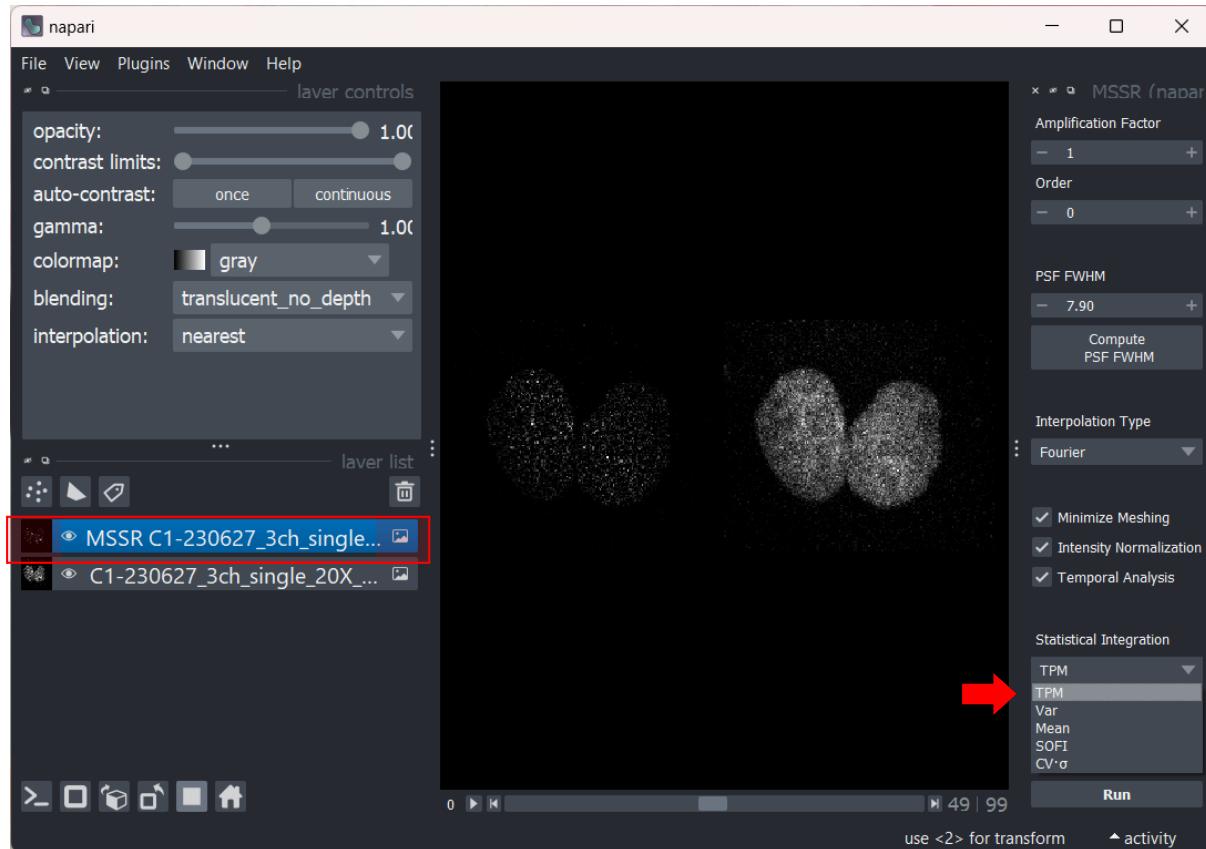


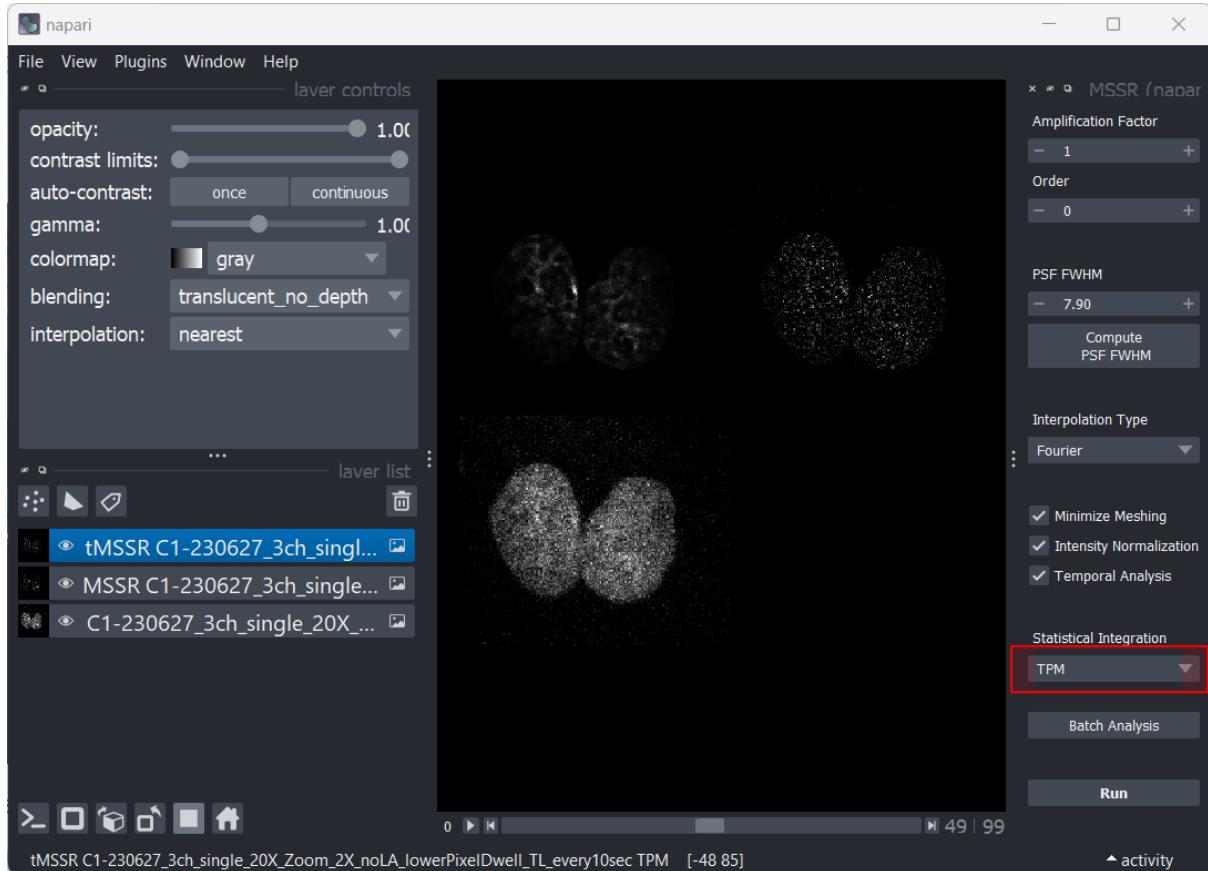
Figure 21. Temporal analysis selection.

- 9) Temporal MSSR analysis.** Select the desired statistical integration from the menu. Then, select the result of the **Single-frame MSSR** analysis (indicated by the red rectangle in fig. 22), and finally, click on "Run."

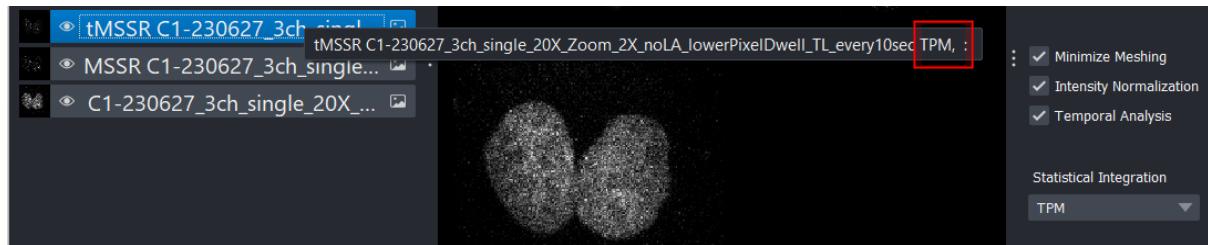


**Figure 22.** Temporal analysis process.

- 10)** The **Temporal analysis** image result will appear in the visualization panel and the file name (blue highlighted text, fig. 23) is placed above the Single-frame MSSR file. The result image will also have the temporal statistical integration mode at the end of its name (fig. 24):



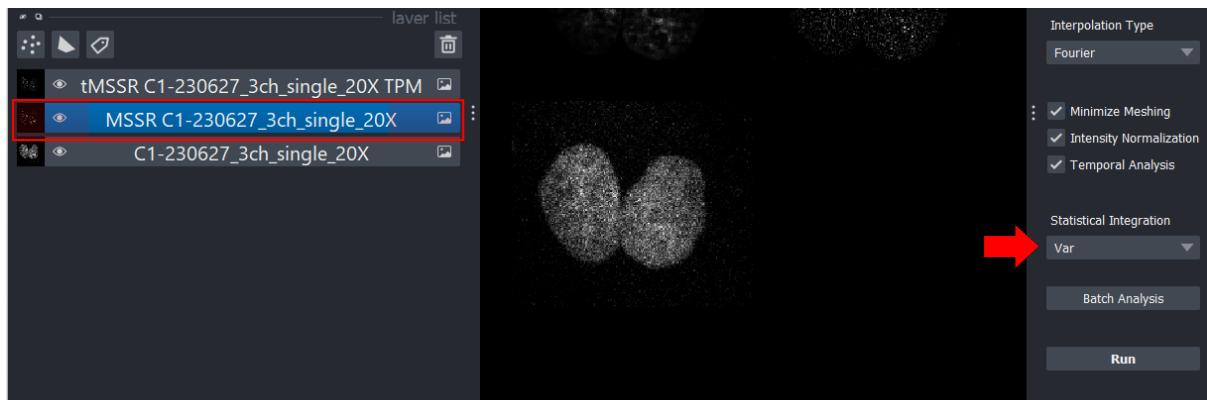
**Figure 23.** Temporal analysis results using the TPM Statistical Integration option.



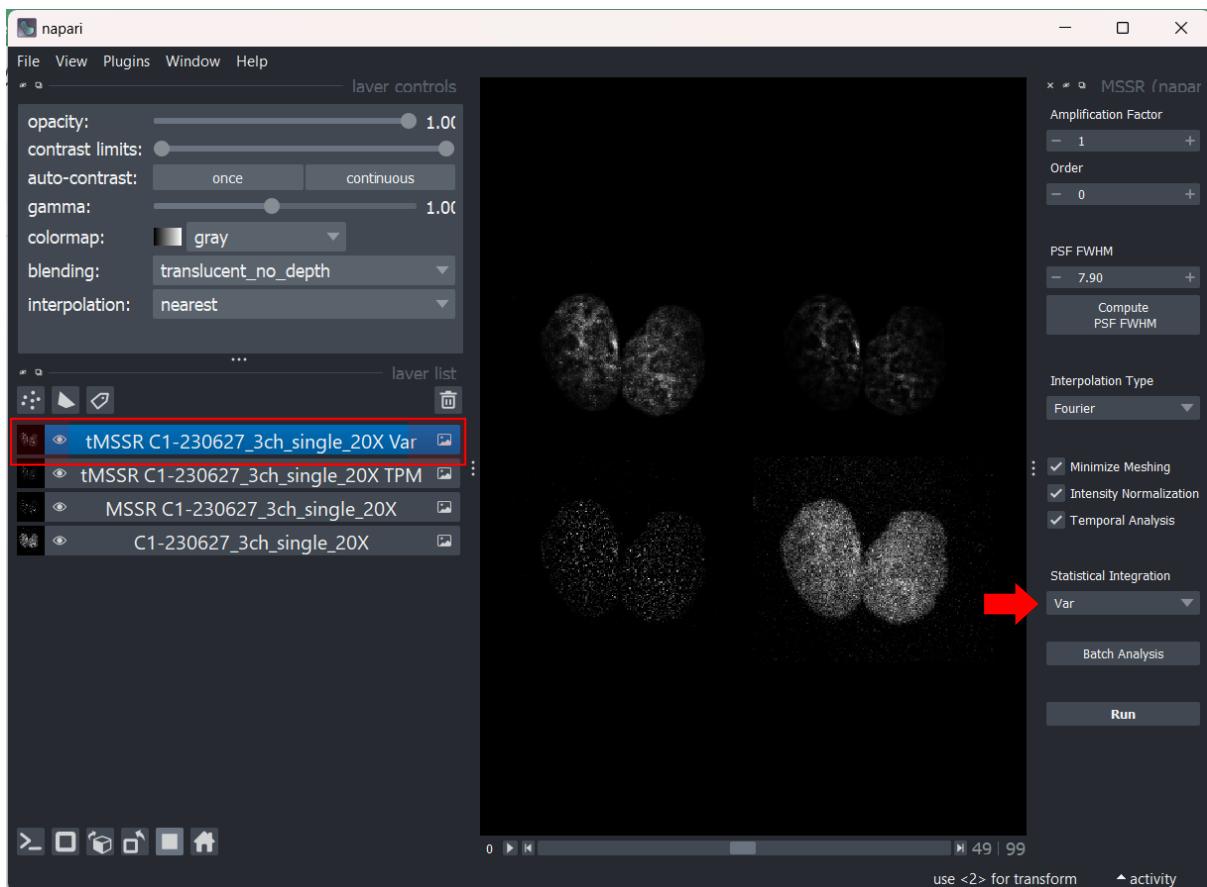
**Figure 24.** The temporal analysis image result indicates the TPM option.

**11) Temporal MSSR analysis.** If you desire to compare between different Statistical Integration options:

1. Change the statistical integration option from the menu (red arrow, fig. 25).
2. Select the Single-frame MSSR analysis result (red rectangle, fig. 25)
3. Finally, click **Run** again, do not forget to click on the Toggle grid mode (Ctrl+G) to visualize all the image results.
4. Napari will display an additional image of the new temporal analysis (fig. 26):

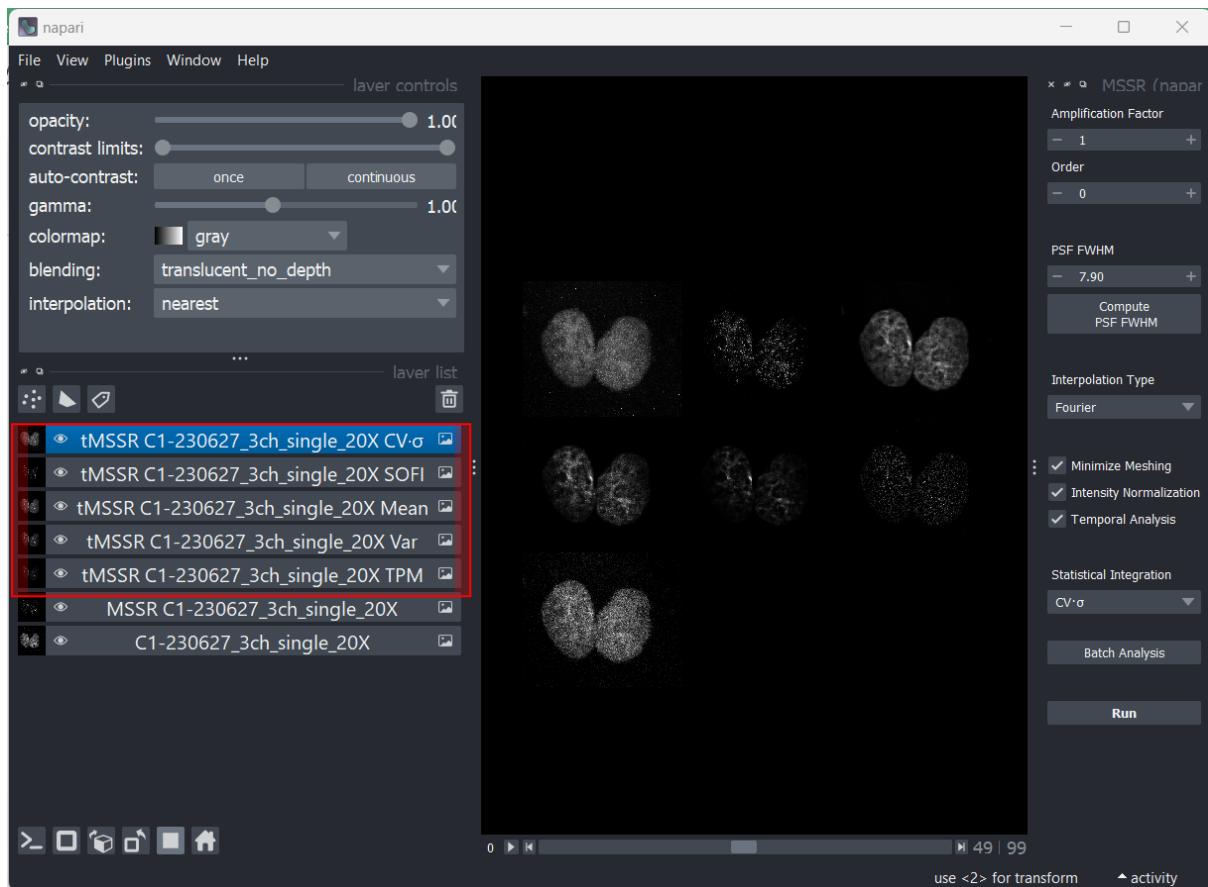


**Figure 25.** Temporal analysis process for using an additional statistical integration option (Var).



**Figure 26.** Temporal analysis results using the Var Statistical Integration option.

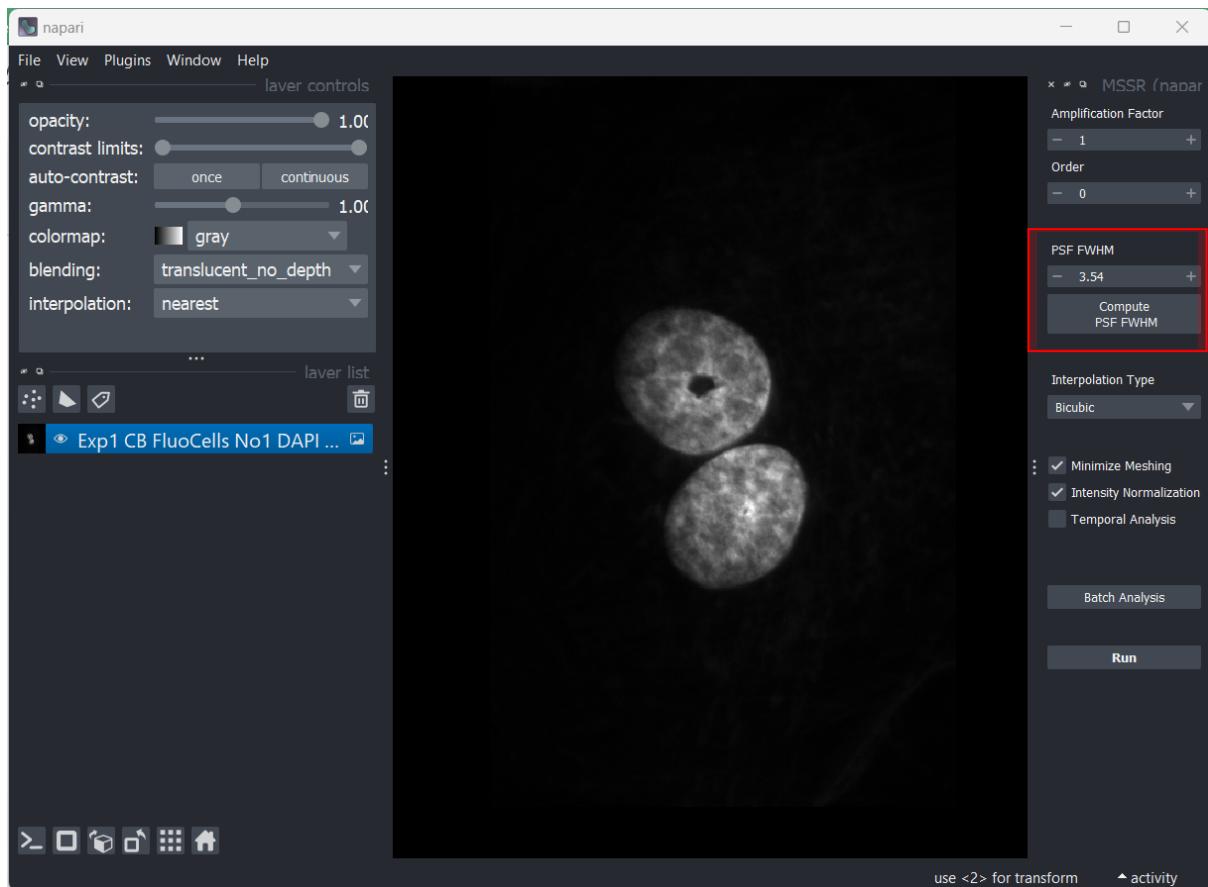
**12) Temporal MSSR analysis.** Compare the results obtained from different Statistical Integration options. Repeat the process of the last step for each Statistical Integration option. Each new image will appear above the single-frame MSSR analysis, marked with the tMSSR tag (indicated by the red rectangle in fig. 27).



**Figure 27.** Temporal analysis: comparison between different Statistical Integration options.

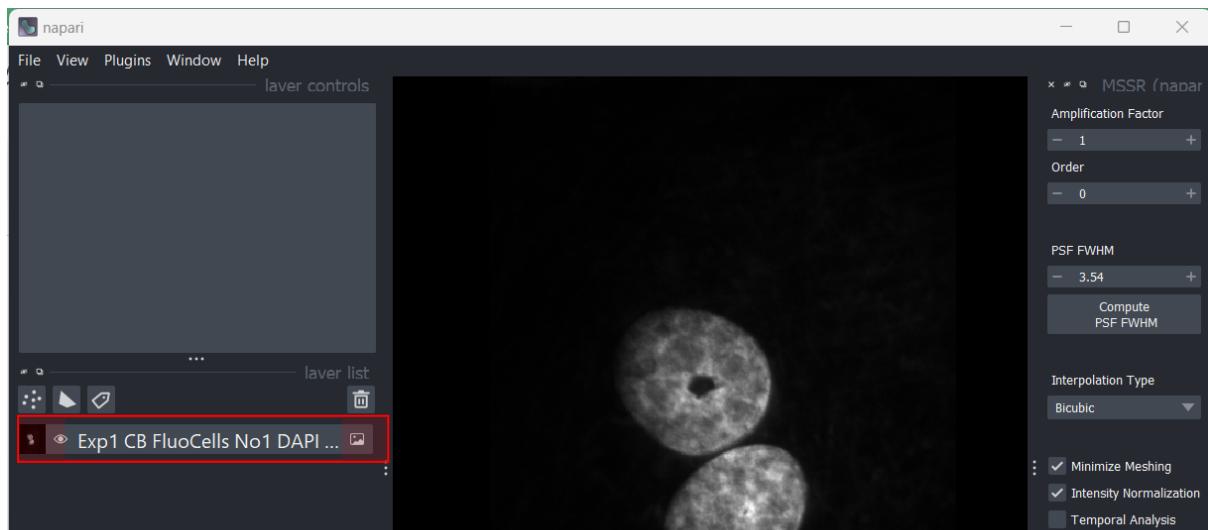
### 3.3 Batch analysis.

**13) Batch analysis:** Open one image from the folder that contains all the images you want to analyze. Utilize the Decorrelation option to calculate the PSF FWHM (fig. 28). Note: It is crucial to ensure that all the other images to be analyzed in this mode were acquired using the same optical parameters. Otherwise, the estimation of the PSF may differ.



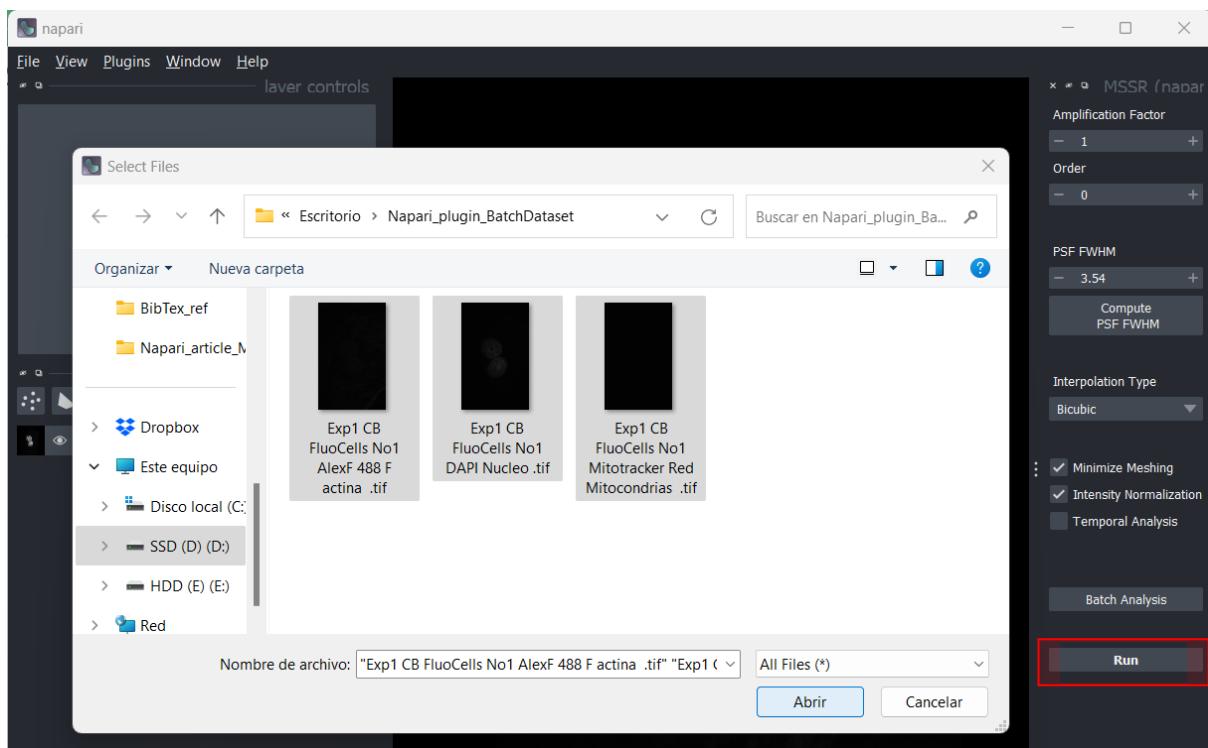
**Figure 28.** Batch analysis: PSF FWHM estimation using the Decorrelation option.

**14) Batch analysis.** After computing the PSF FWHM, it's crucial that you delete or deselect the image you used to obtain the PSF FWHM estimation (indicated by the red rectangle in the fig. 29 and the text it's no longer blue highlighted):



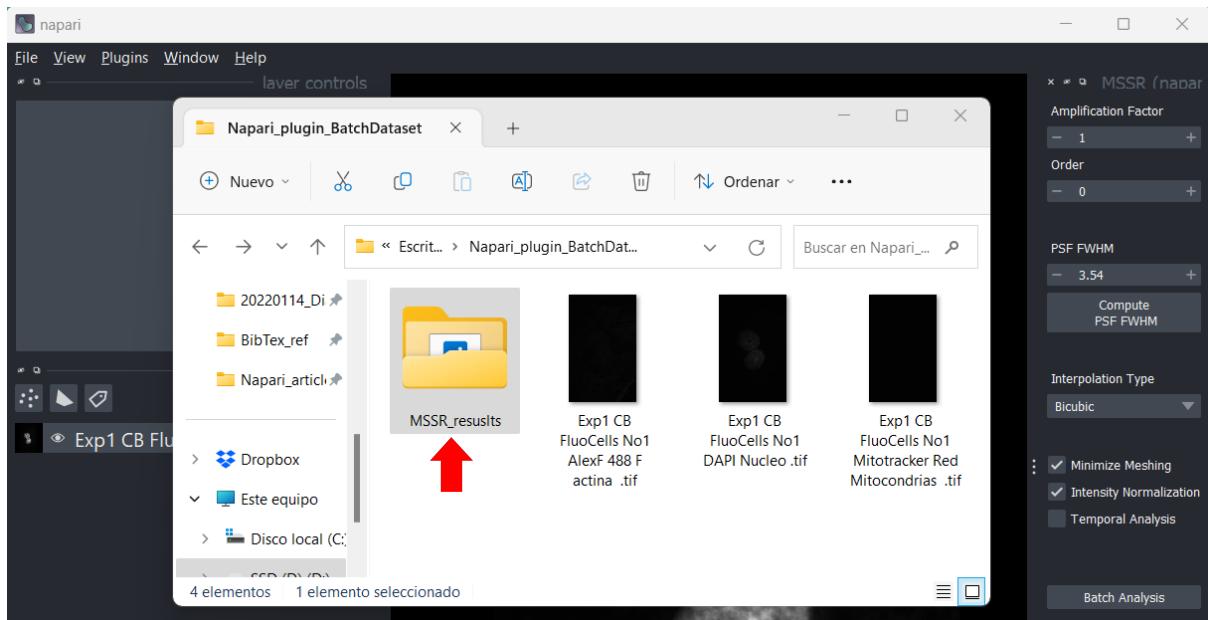
**Figure 29.** Deselect the image used to estimate the PSF FWHM.

**15)** Then, select the "**Batch Analysis**" option from the MSSR menu. Choose the images you wish to analyze and click "**Run**" (fig. 30).



**Figure 30.** Select the images to be analyzed by the Batch Analysis option.

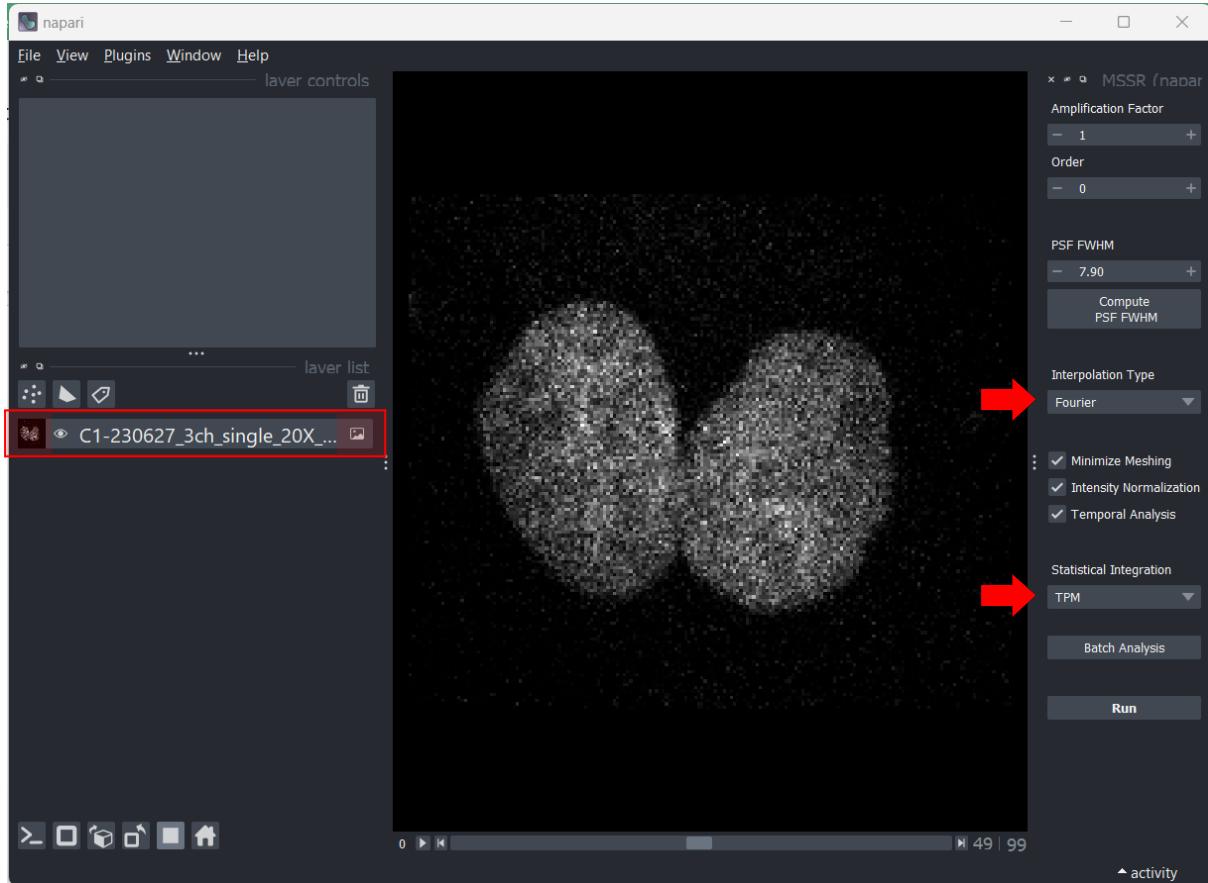
- 16)** The MSSR single-image analysis results will appear in a specific folder named “MSSR\_results” contain in the same folder where the original images are placed (fig. 31):



**Figure 31.** Batch Analysis results indicated by a red arrow.

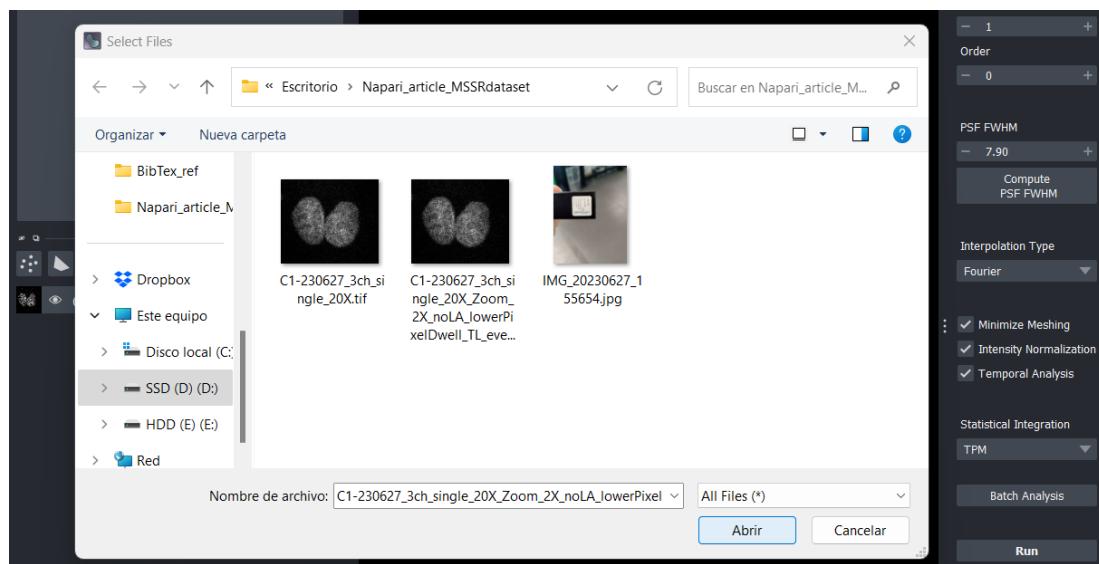
- 17) Batch analysis.** It is your choice whether you want to perform a single-image analysis (as the example explained before) or a temporal analysis, depending on the type of files you have. If you have stacks of images, you can select them and then proceed to click on the temporal analysis and statistical integration options, as explained earlier.

Note: Do not forget to estimate the PSF FWHM and complete all the required parameters first and then perform the Batch Analysis.



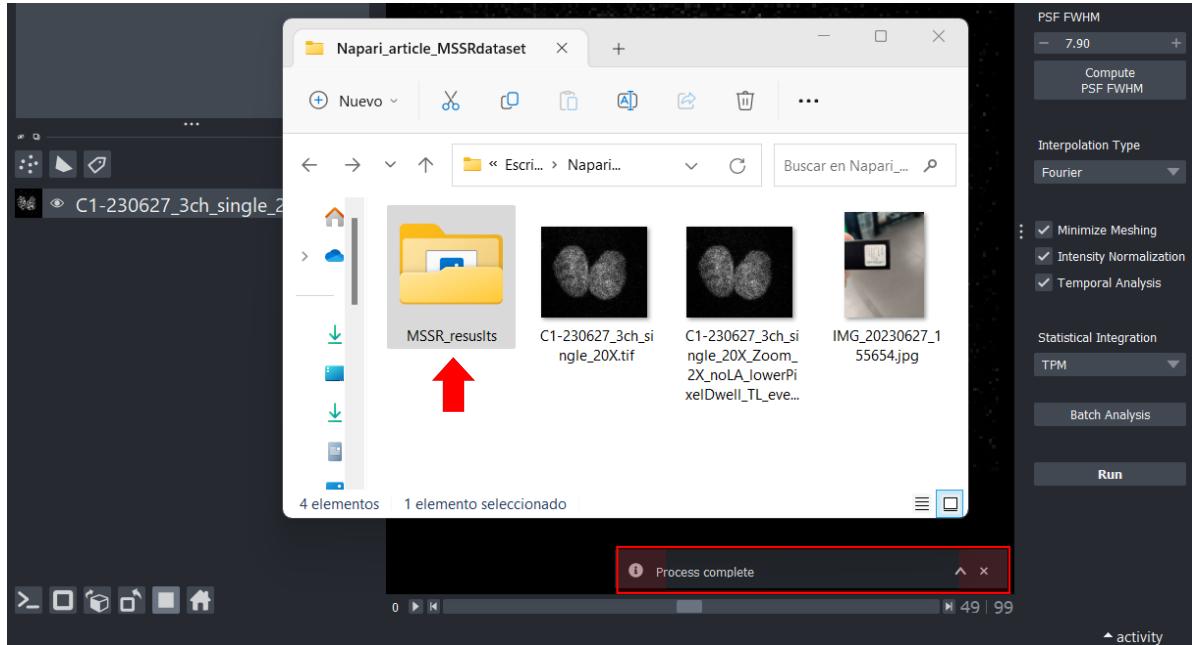
**Figure 32.** PSF FWHM estimation of a stack of images. Red rectangle represents the stack (99 images), do not forget to deselect it after the PSF FWHM estimation. Red arrows indicate the change in the Interpolation Type and the Statistical Integration in order to perform a temporal analysis through the Batch Analysis option.

- 18)** After the PSF FWHM estimation, click on the “Batch Analysis” option and select the desired files from a folder. Finally, click on “Run”.



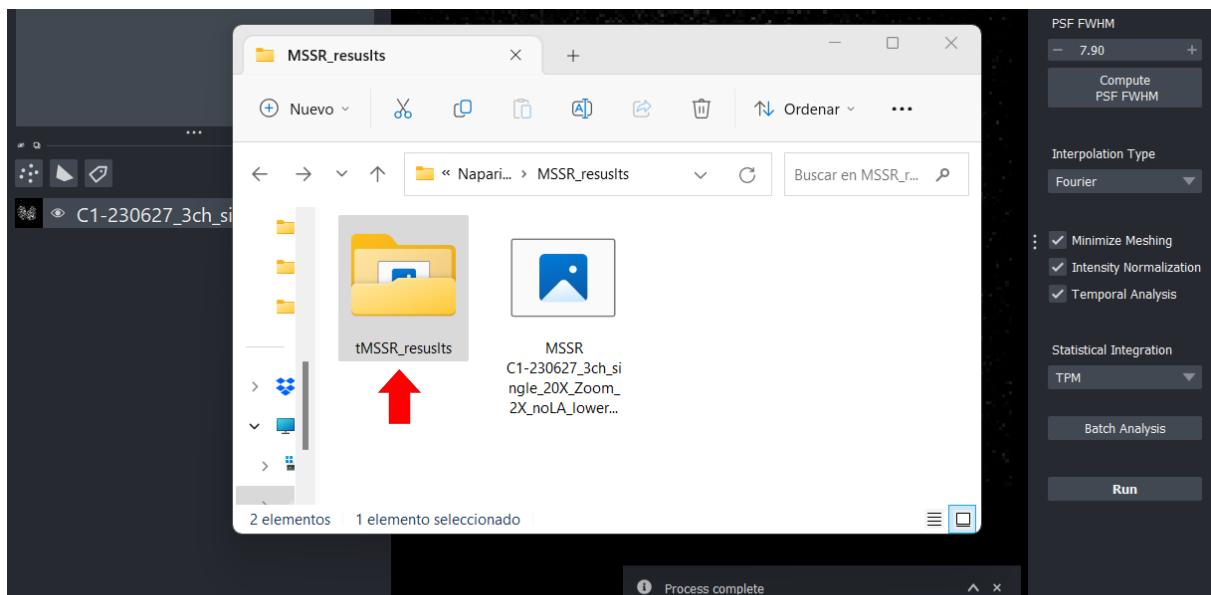
**Figure 32.** Stack of images selection to perform a temporal analysis through the Batch Analysis option.

**19)** Once the process has been completed (indicated by the red rectangle, fig. 33) the MSSR plugin will provide you with the single-image results and then a new folder with the temporal analysis results will appear automatically on your file explorer:



**Figure 33.** MSSR results from the Batch Analysis option of the temporal analysis of a stack of images.

**20)** Inside the folder named “**MSSR\_results**” you will find the single-frame analysis of the stack and another folder with the temporal analysis named “**tMSSR\_results**” (indicated by a red arrow, fig. 34). Note: if you need to perform another temporal analysis with different parameters you have to replace this folder first or rename it.



**Figure 34.** tMSSR results from the Batch Analysis option.

## **4. ESI MANUAL**

ESI is a technique that measures the amount of information in a series of images where the brightness changes or blinks, like in experiments where single molecules are located. To create a more detailed image from the overall fluorescence data, we use a calculation that combines Shannon-Entropy (a measure of information content) with higher-order statistics (more complex statistical calculations) on each pixel of the image. This helps us reconstruct a higher-resolution image from the original widefield fluorescence data.

To apply the ESI algorithm, some parameters are required:

### **4.1 Number of images in output.**

Refers to the resulting number of images that are reconstructed or generated after applying the ESI algorithm to the input image sequences.

ESI uses an iterative process, where an initial stack of image frames is divided into subsets or substacks. Each substack is processed separately, resulting in a set of reconstructed frames.

So the parameter determines how many of these reconstructed images will be generated as a result.

### **4.2 Number of bins for entropy.**

Refers to the number of categories or divisions used to calculate entropy.

Entropy, in this context, is a measure of the amount of information or randomness in an image.

Parameter determines how finely the range of pixel intensities is divided or categorized when calculating entropy. It represents the number of distinct intensity levels or intervals considered in the analysis, ie, determines the level of granularity or detail in the calculation of entropy, influencing the resolution enhancement achieved by the ESI algorithm.

### **4.3 Order.**

Refers to the order of the statistical moments that are mathematical values that describe different properties of a distribution of data. They provide information about the shape, spread, and other characteristics of a dataset and are used in the calculation of entropy-weighted higher-order statistics.

The order of the moment determines which specific property of the distribution is being considered.

Using higher-order moments in the calculation allows for a more refined analysis of the data, enabling the reconstruction of high-resolution images with finer details.

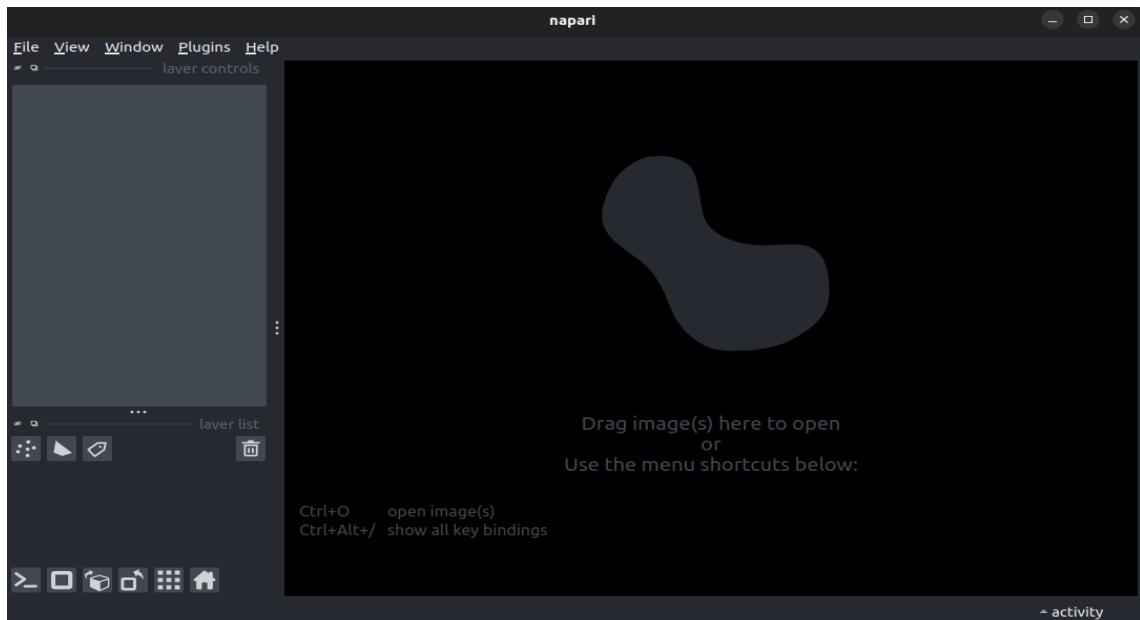
### **4.4 Intensity Normalization.**

Refers to a parameter that is used to adjust the pixel intensities in the image data before applying the ESI algorithm, is a process of rescaling or adjusting the intensity values of pixels in an image to a common range or distribution. This normalization step is often

performed to ensure that the pixel intensities are within a desired range or have a specific statistical property, which can improve the effectiveness of subsequent image processing algorithms.

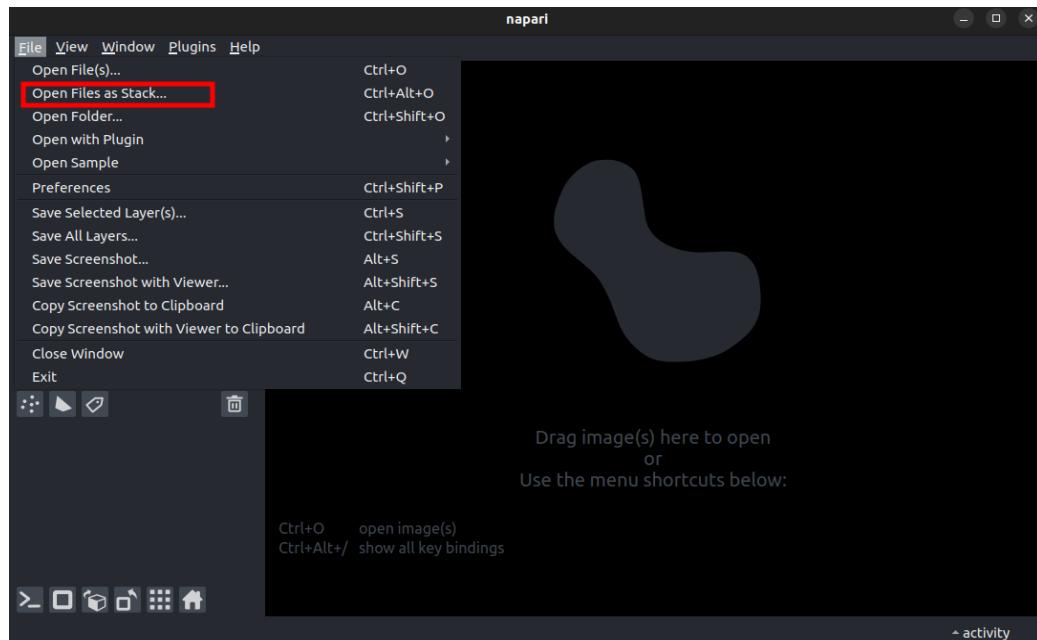
Once the parameters are understood, let's see how to apply ESI using napari.

When starting napari, the first screen that will be displayed to us is the following:



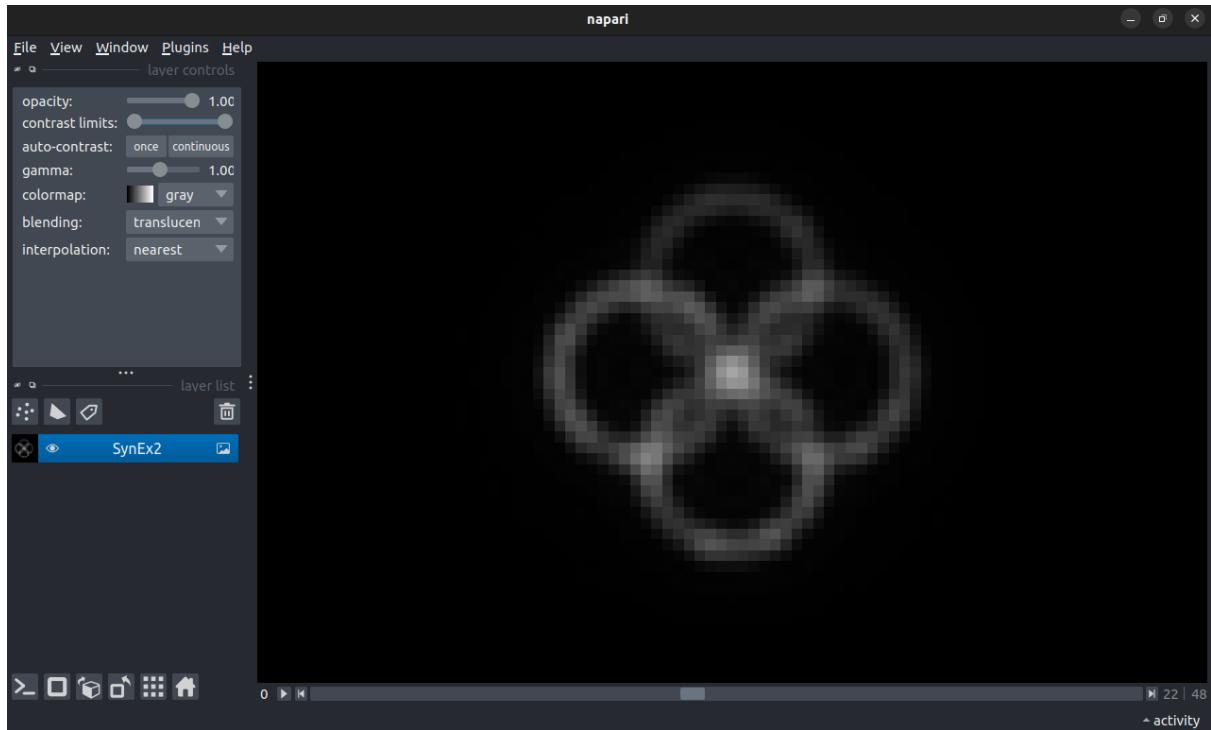
**Figure 35.** Main screen of napari.

Click on the "File" tab of the program to load the stack, which consists of 49 images in this case.



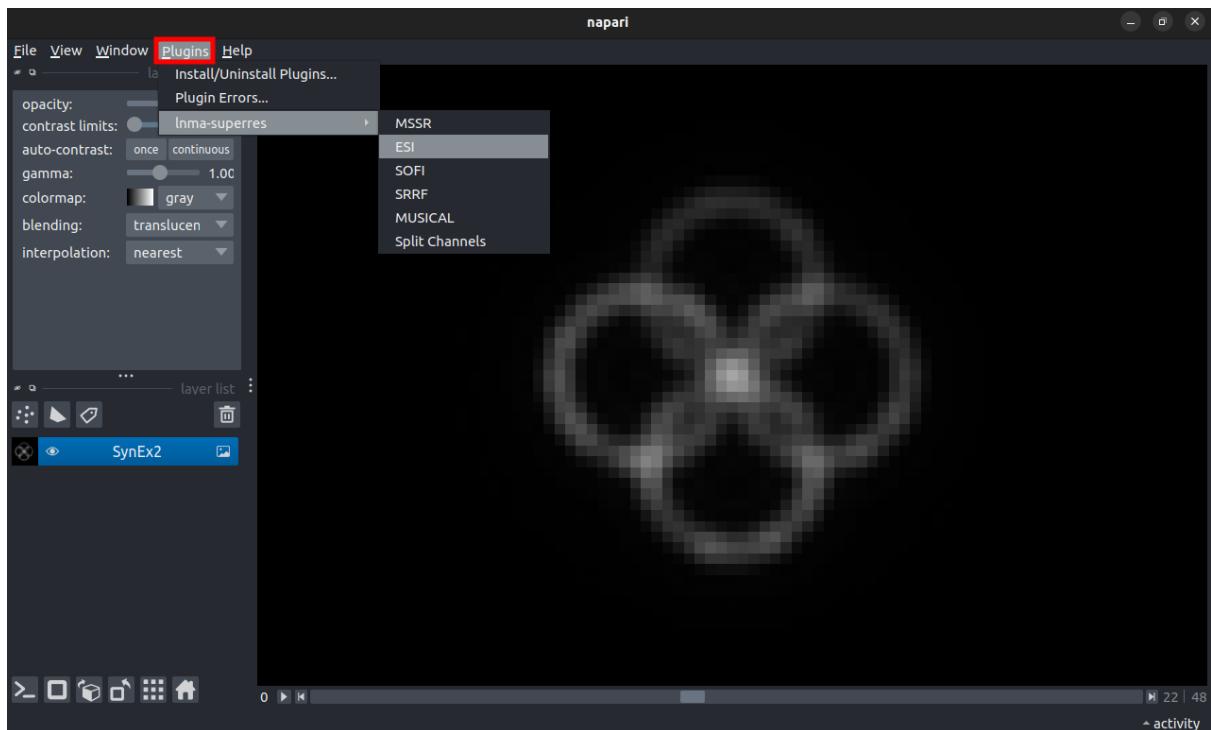
**Figure 36.** Option in the napari menu to open images (red rectangle).

Search for the dataset within the computer's file system, when opening the dataset, Napari will display a visualization.



**Figure 37.** After opening the image, it can be viewed in napari.

In order to apply ESI, we click on the "Plugins" tab and we navigate to the "Inma-superres" section where we find the "ESI" option.



**Figure 38.** Option for plugins in the napari menu (red rectangle).

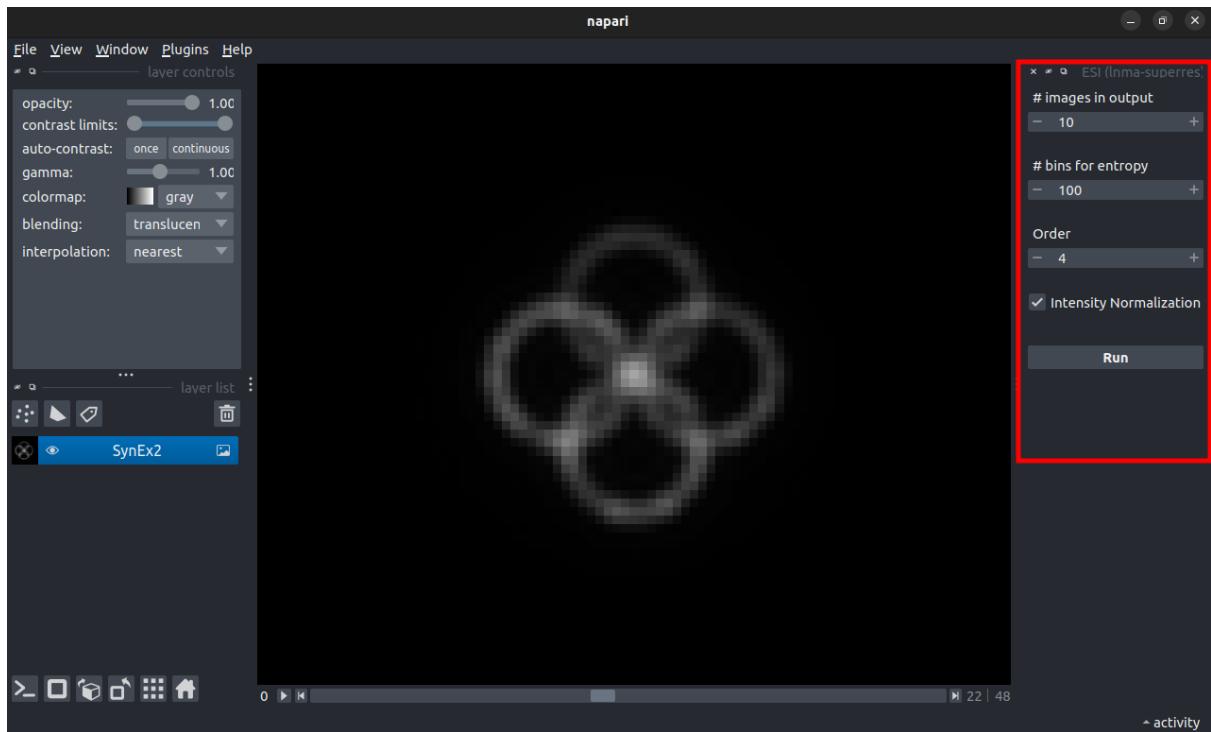
By selecting the algorithm on the right side of the screen, a panel opens where we can define the parameters described earlier. In this case, we will use the following values.

Number of images in output: 10

Number of bins for entropy: 100

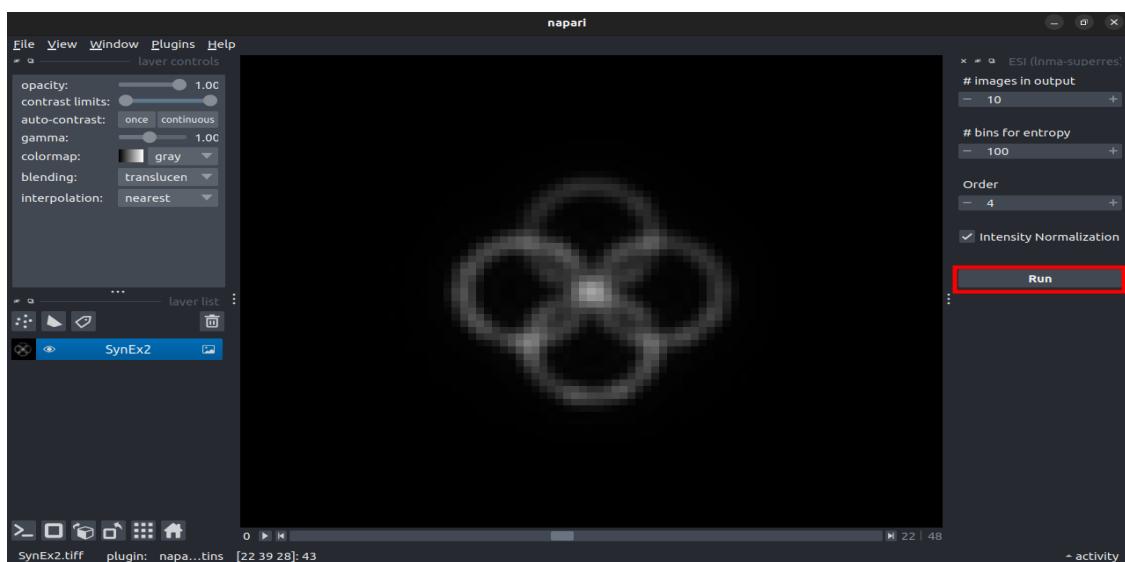
Order: 4

Checked Intensity normalization



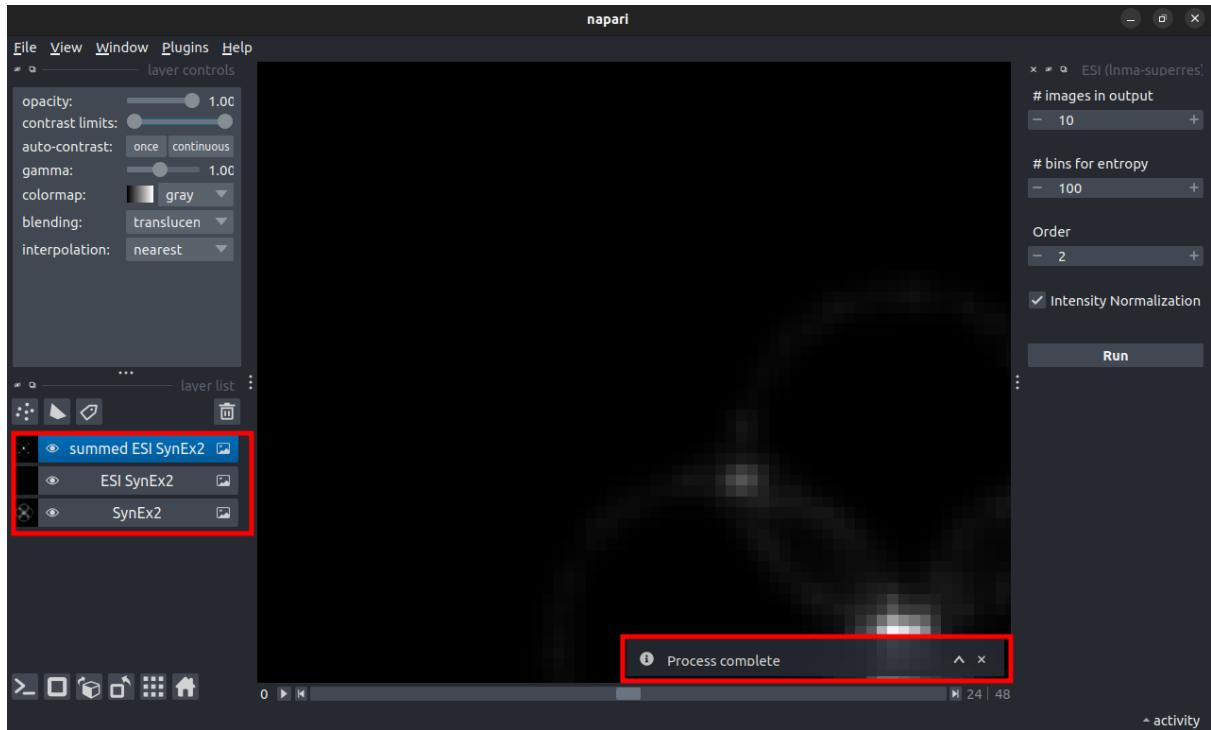
**Figure 39.** Parameter selection panel for ESI application (red rectangle).

We apply the algorithm by clicking on the "Run" button.



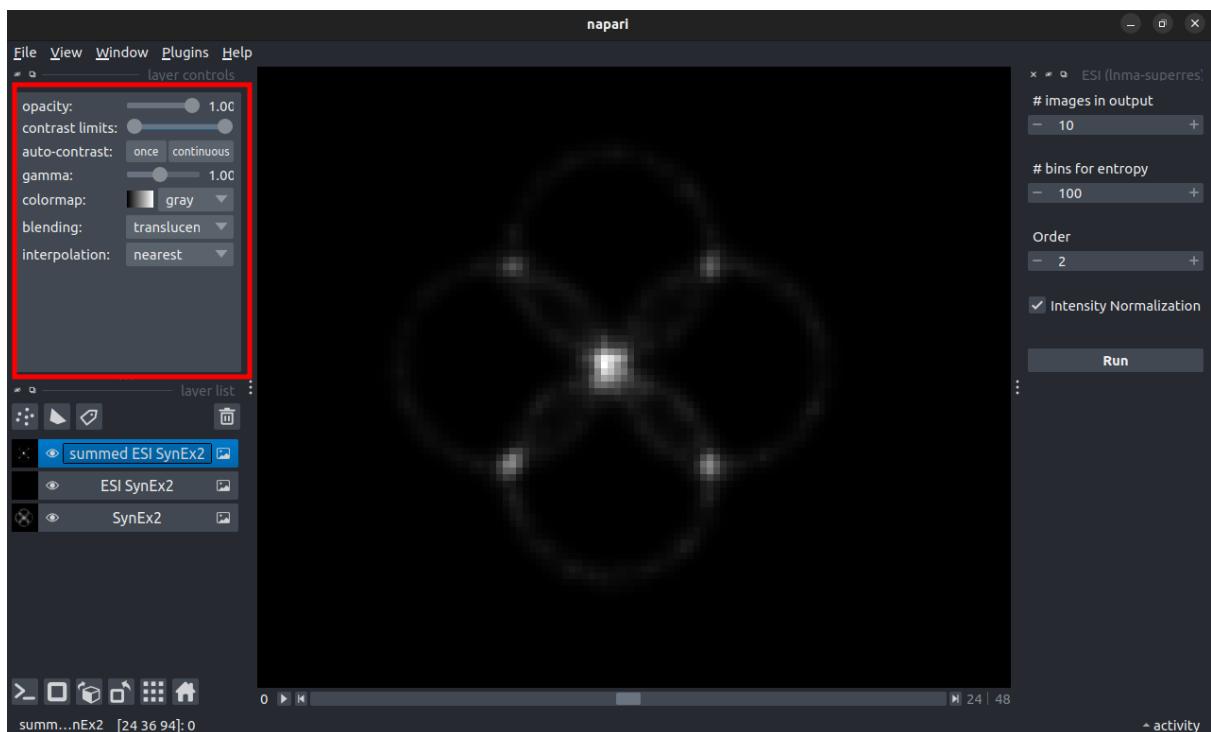
**Figure 40.** Button to execute the ESI method (red rectangle).

Upon completion of the process, we will see a notification indicating that the algorithm has finished, and we will be able to view a visualization of the results.

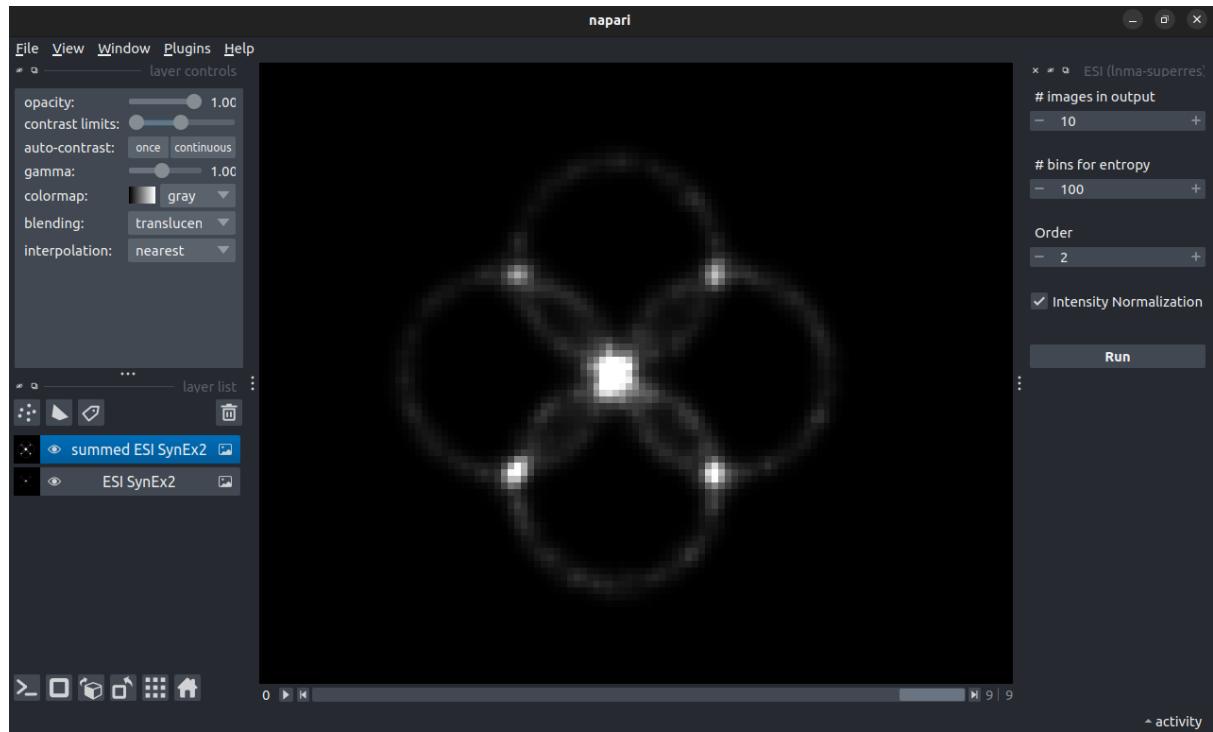


**Figure 41.** Result viewer of the method and notification of method completion (red rectangles).

We can adjust the settings to visualize our processed image.



**Figure 42.** Configuration panel for visualization (red rectangle).



**Figure 43.** Final visualization of the results after applying ESI.

## **5. MUSICAL MANUAL**

Multiple Signal Classification Algorithm (MUSICAL) for images is a technique used to identify and classify different signals or components within an image.

MUSICAL analyzes the statistical relationships between pixels in an image, decomposes the covariance matrix to estimate signal directions, calculates the spatial spectrum of the image, and then classifies the signals based on their characteristics.

To apply the MUSICAL algorithm, some parameters are required:

### **5.1 Emission $\lambda$ [nm].**

Refers to the wavelength of the fluorescence emission from fluorophores in nanometers.

Is important because it helps determine the resolution and quality of the super-resolution images obtained, so by knowing the wavelength, the algorithm can take into account this information and use it in combination with other factors to improve the spatial resolution of the final reconstructed image.

### **5.2 Numerical Aperture.**

Refers to a characteristic of the microscope objective lens used in fluorescence microscopy.

Represents the ability of the lens to gather and focus light. It is determined by the lens design and is typically represented by a numerical value.

A higher Numerical Aperture means that the lens can gather more light and focus it to a smaller spot, resulting in improved resolution and greater ability to capture fine details.

### **5.3 Magnification.**

Refers to the factor by which the size of an object or image is increased when observed through a microscope.

Is important because it determines the scale at which the super-resolution images are reconstructed. By knowing the magnification factor, the algorithm can accurately map the observed features and structures to their corresponding size in the original sample.

### **5.4 Pixel size [nm].**

Refers to the physical size in nanometers of each individual pixel in the digital image captured by the camera or detector during the imaging process.

Is important because it determines the level of detail that can be captured and resolved in the final reconstructed image.

### **5.5 Plot Singular Values.**

Involves analyzing and graphically representing the importance or significance of different patterns or structures in the image data.

Provides a visual representation of the importance or contribution of each pattern or structure to the overall image. Larger singular values indicate patterns that are more prominent and influential, while smaller singular values represent less significant patterns or noise.

### **5.6 Threshold.**

Refers to a value that is used to distinguish between meaningful signal and noise in the image data.

Is used as a criterion to determine which patterns or signals are considered significant and should be included in the final reconstruction.

It affects the accuracy and quality of the reconstructed super-resolution image.

### **5.7 Alpha.**

Is used in the computation of an indicator function that helps determine the presence or absence of fluorophores or desired structures in the image.

Choosing an appropriate value for alpha is crucial because it affects the trade-off between resolution enhancement and noise suppression. A higher value of alpha increases the emphasis on closely located test points, potentially enhancing resolution but also increasing the risk of including more noise. On the other hand, a lower value of alpha reduces the emphasis on closely located test points, which may lead to less resolution improvement but also less noise.

### **5.8 Subpixels per pixel.**

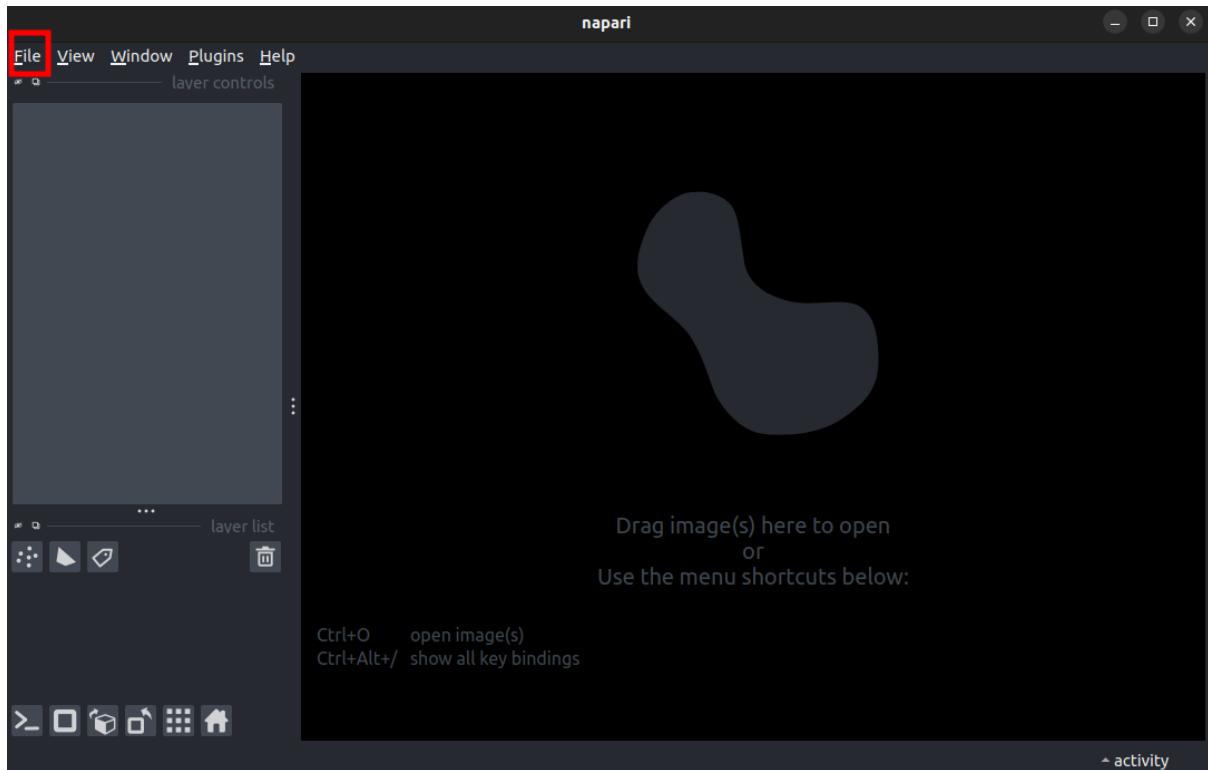
Refers to the level of subdivision or granularity used to enhance the resolution of the reconstructed image.

These subpixels represent smaller divisions within each pixel of the original image. By subdividing each pixel into multiple subpixels, MUSICAL can capture more detailed information and enhance the resolution of the final reconstructed image.

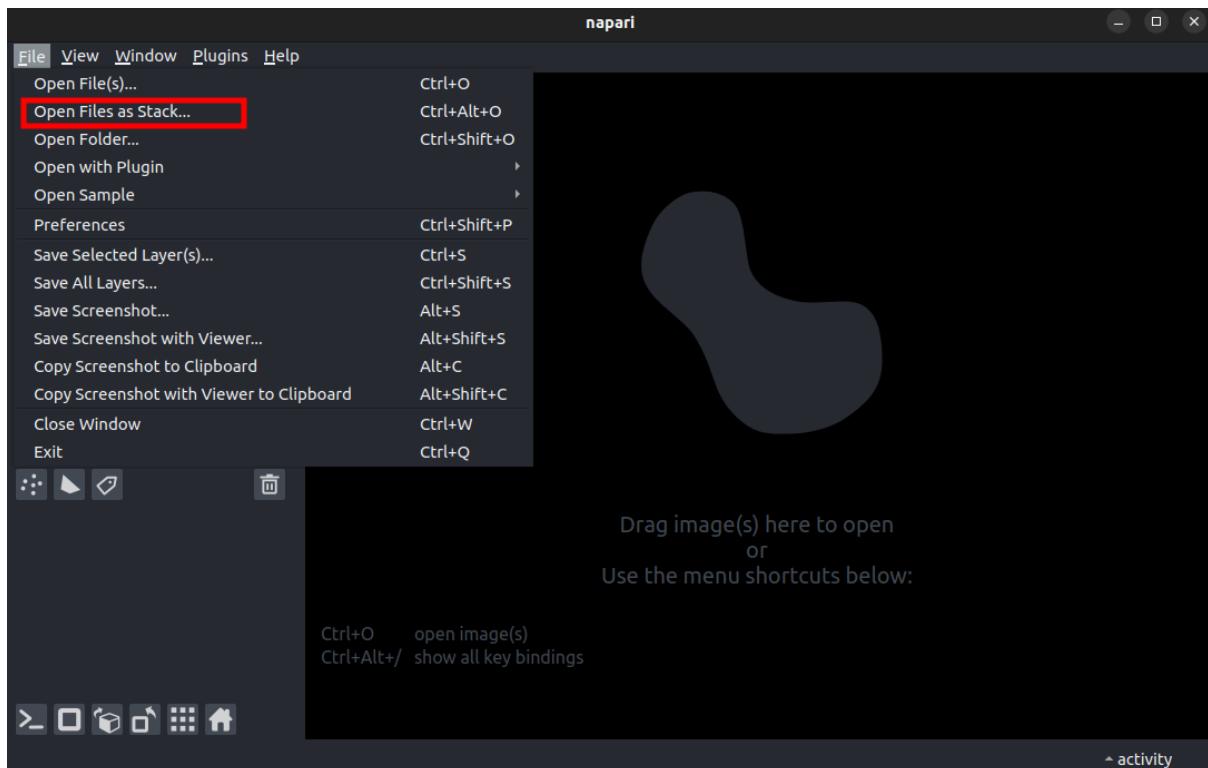
Determines the number of subdivisions or sub pixels within each pixel. A higher value means more subpixels are used, resulting in finer detail and potentially higher resolution in the reconstructed image. Conversely, a lower value means fewer subpixels are used, resulting in less detail and potentially lower resolution.

Once the parameters are understood, let's see how to apply MUSICAL using napari.

Click on the "File" tab of napari to load the stack, which consists of 100 images in this case.

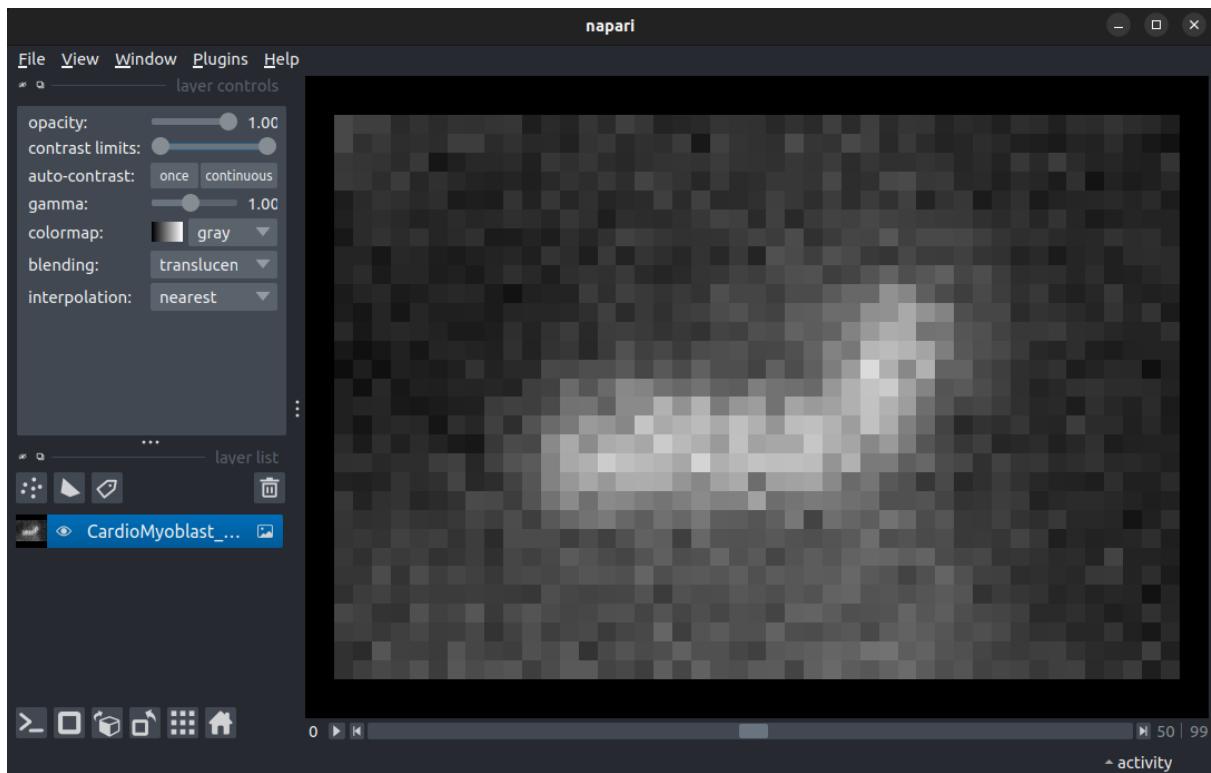


**Figure 44.** Option in the napari menu to files (red rectangle).



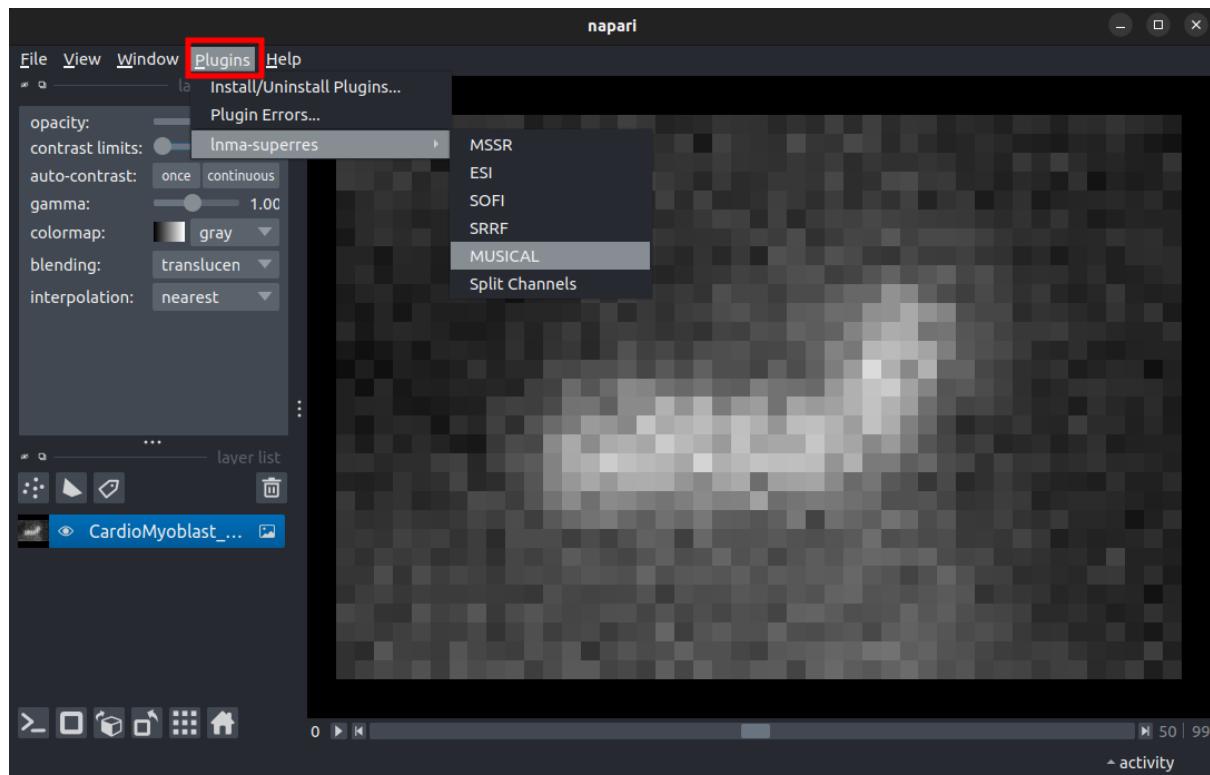
**Figure 45.** Option in the napari menu to open images (red rectangle).

Search for the dataset within the computer's file system, when opening the dataset, Napari will display a visualization.



**Figure 46.** After opening the image, it can be viewed in napari.

In order to apply MUSICAL, we click on the "Plugins" tab and we navigate to the "Inma-superres" section where we find the "MUSICAL" option.



**Figure 47.** Option for plugins in the napari menu (red rectangle).

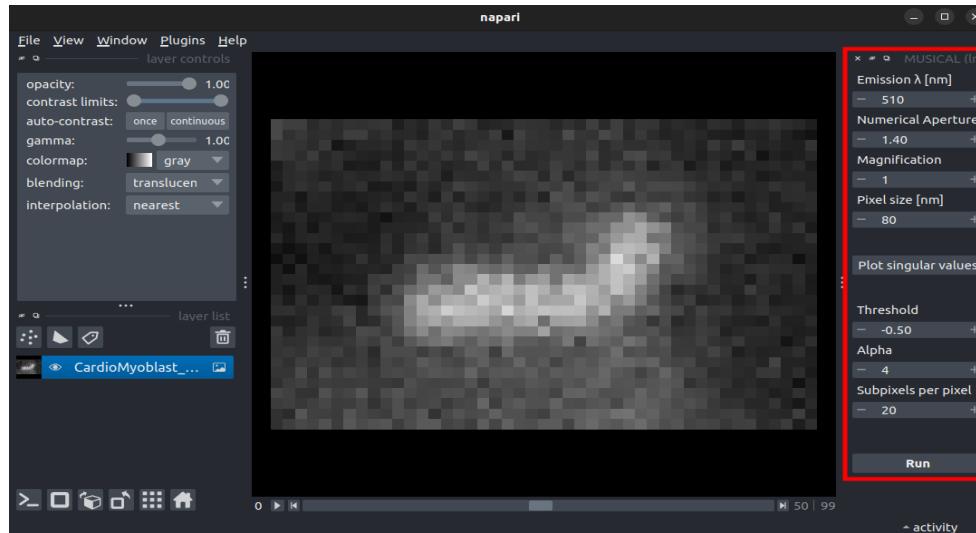
By selecting the algorithm on the right side of the screen, a panel opens where we can define the parameters described earlier. In this case, we will use the following values.

Emission [nm]: 510

Numerical aperture: 1.4

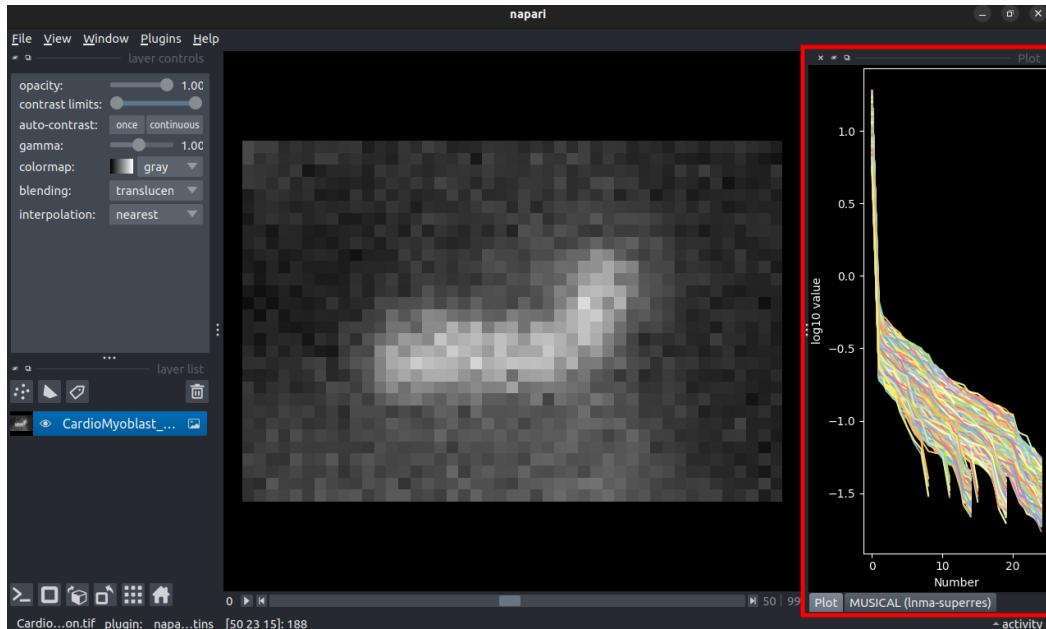
Magnification: 1

Pixel size [nm]: 80



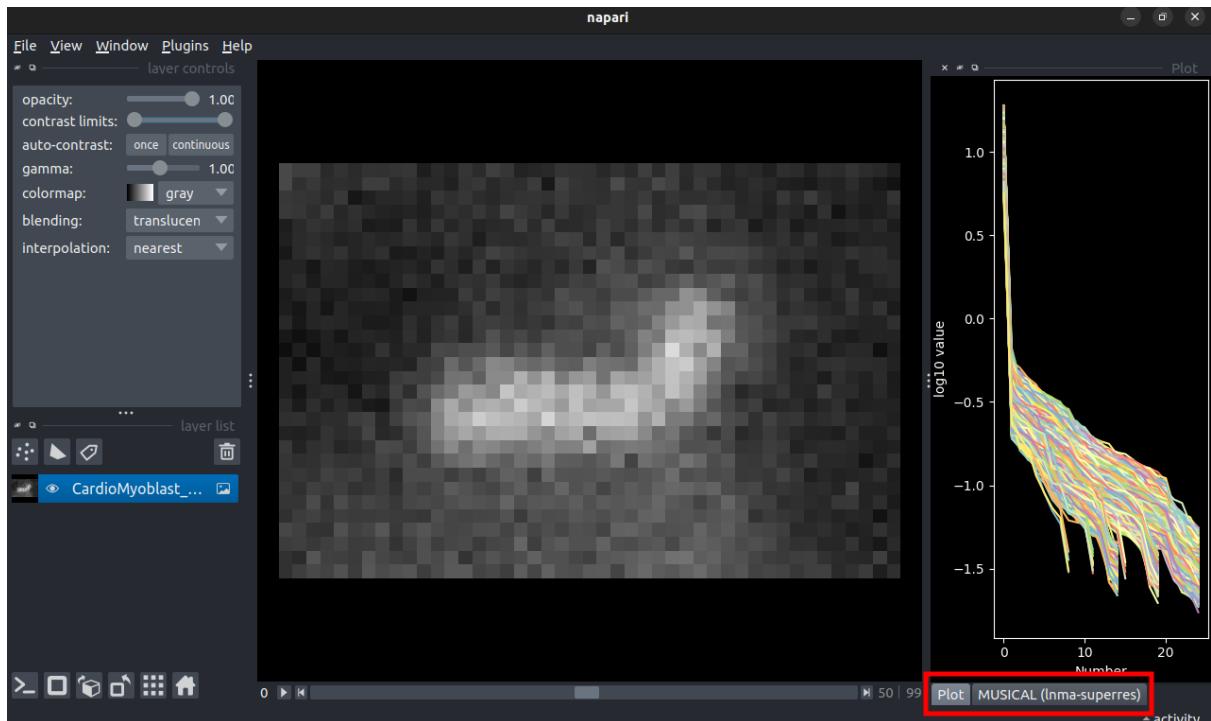
**Figure 48.** Option for plugins in the napari menu (red rectangle).

If we click on "Plot singular values," it will generate a graph in the right corner of the screen that allows us to visualize these values.



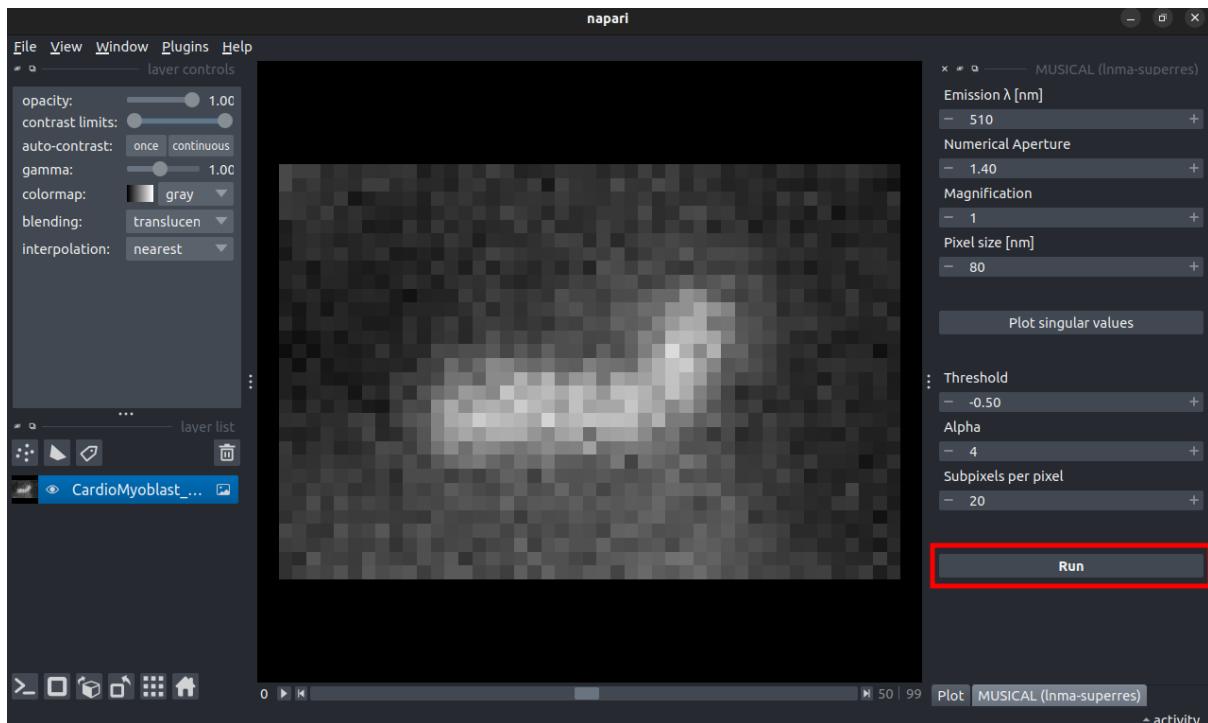
**Figure 49.** Singular value plot viewer (red rectangle).

We can navigate between the graph visualization and the parameters using the buttons located in the bottom right corner.



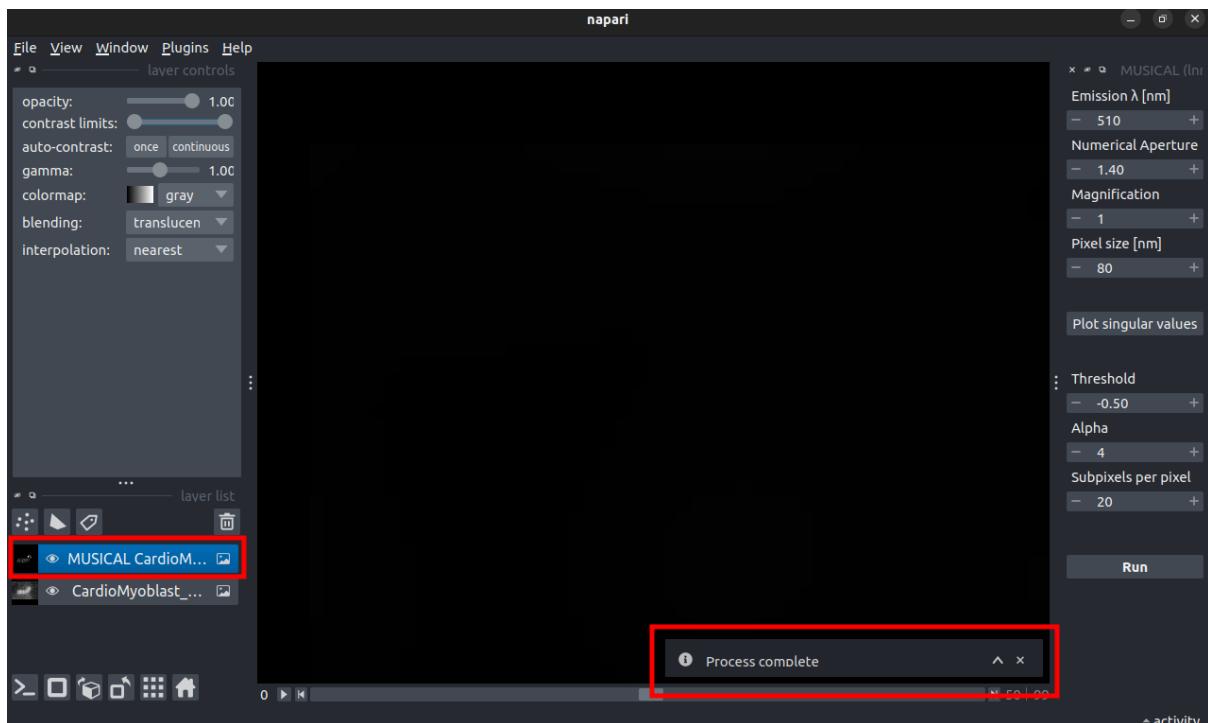
**Figure 50.** Tabs to navigate between the graph view and the method parameters (red rectangle).

We apply the algorithm by clicking on the "Run" button.



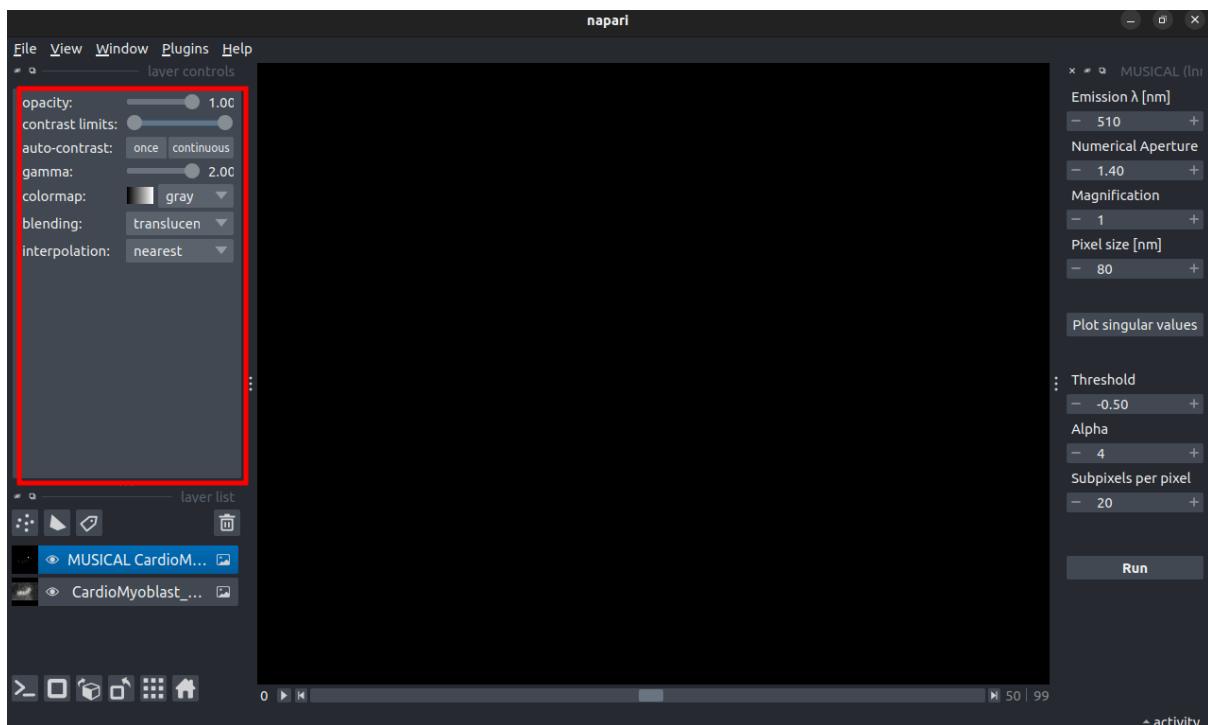
**Figure 51.** Button to execute the MUSICAL method (red rectangle).

Upon completion of the process, we will see a notification indicating that the algorithm has finished, and we will be able to view a visualization of the results .

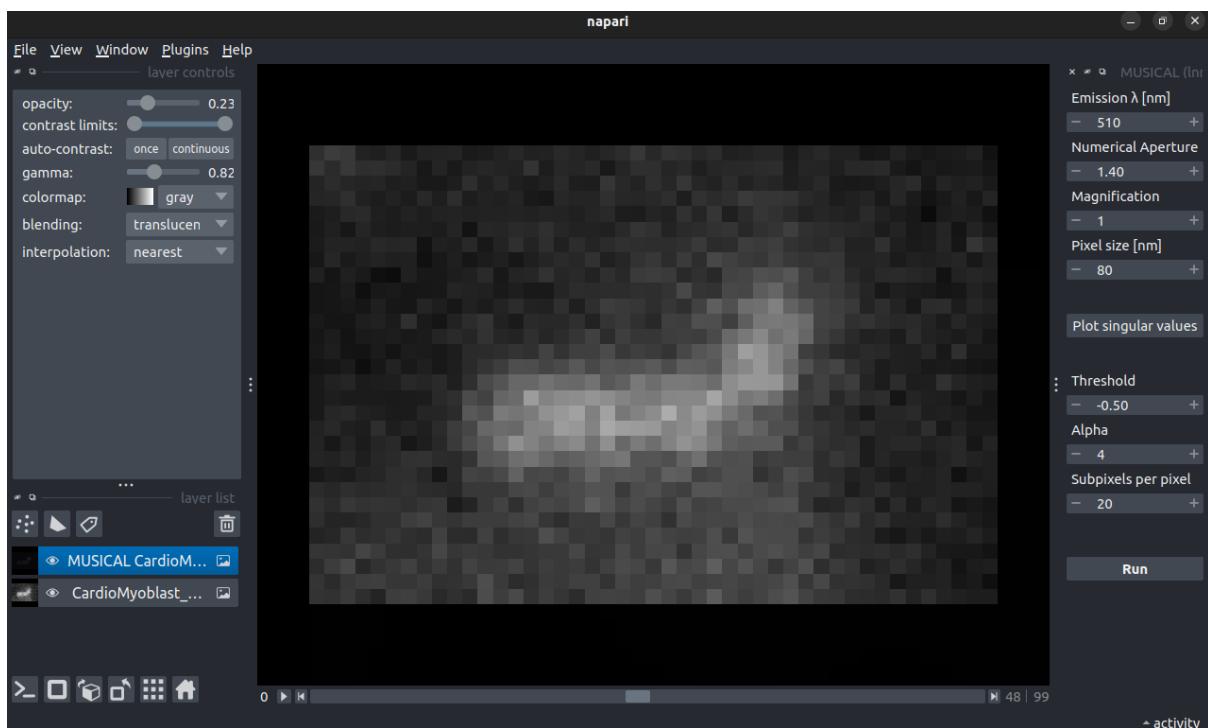


**Figure 52.** Result viewer of the method and notification of method completion (red rectangles).

We can adjust the settings to visualize our processed image.



**Figure 53.** Configuration panel for visualization (red rectangle).



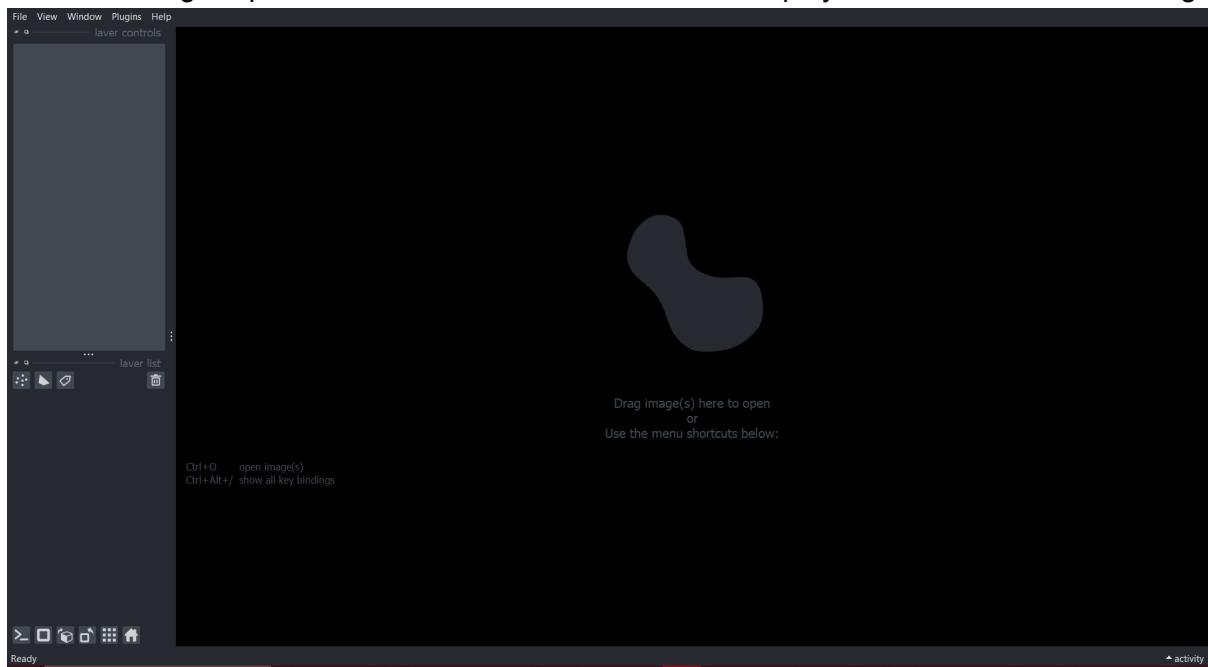
**Figure 54.** Final visualization of the results after applying MUSICAL.

## **6. SRRF MANUAL**

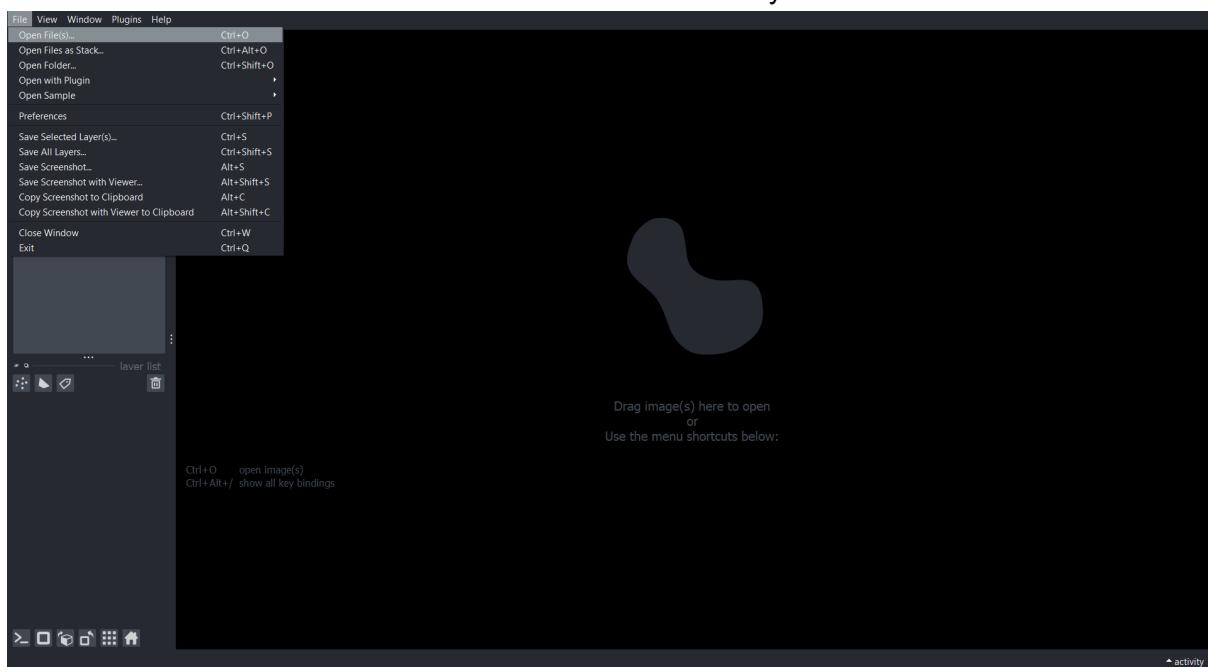
SRRF (Super-resolution Radial Fluctuations) is an advanced algorithm used for super-resolution imaging. It leverages the analysis of radial and temporal fluctuations in fluorescence intensity within a sequence of images. By combining spatial and temporal information from multiple frames, SRRF can generate high-resolution images with remarkable detail, reaching an impressive resolution of approximately 60 nm. The underlying principle behind SURF's effectiveness lies in the distinction between noise, which lacks temporal correlation, and fluorophores, which exhibit temporal correlations due to their inherent properties.

Now, let's explore the process of applying SRRF using napari. We will also delve into understanding the significance of each parameter involved in the SRRF algorithm.

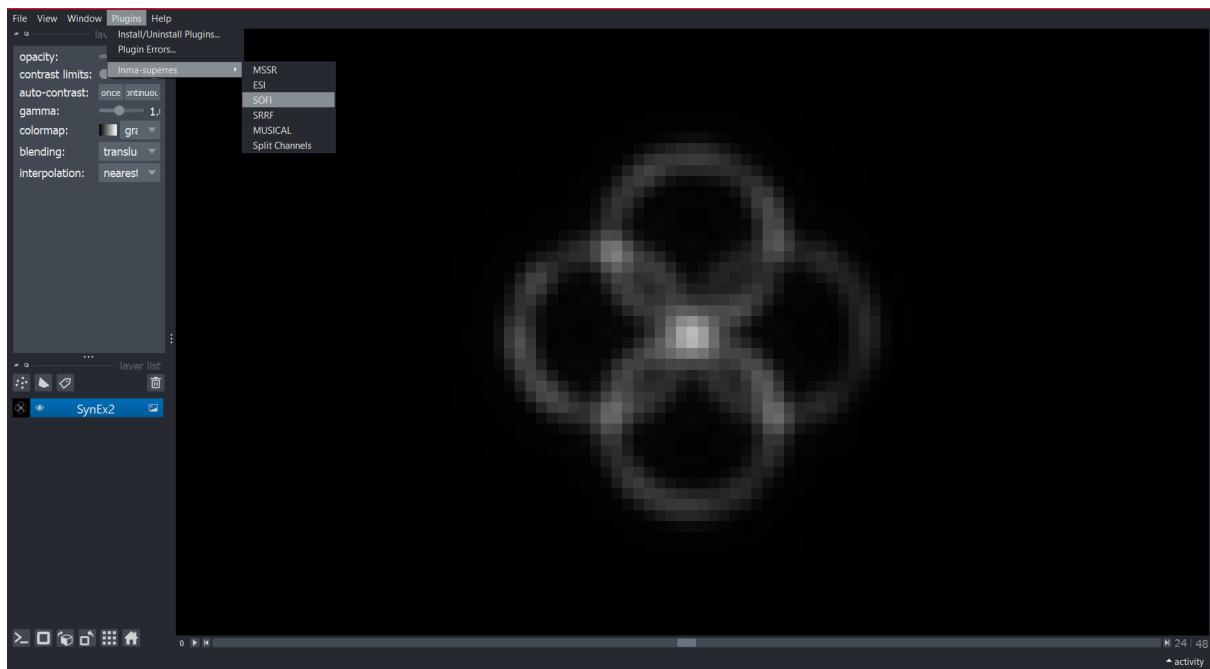
When starting napari, the first screen that will be displayed to us is the following:



To open an image stack, you need to go to the "File" section and then select "Open File(s)".  
And select your data.



Once you have loaded your files, you will navigate to the settings. Plugins > Inma-superres > SRRF



When you select the SRRF plugin, a new console will appear on the left side with the following options. Below, we will describe each of the parameters necessary for the analysis.

## 6.1 Amplification factor.

The amplification factor determines the number of subpixels into which the original image pixels are divided. A higher number of subpixels leads to improved resolution. However, it is important to note that increasing the number of subpixels also increases the computational

time required to run the algorithm.



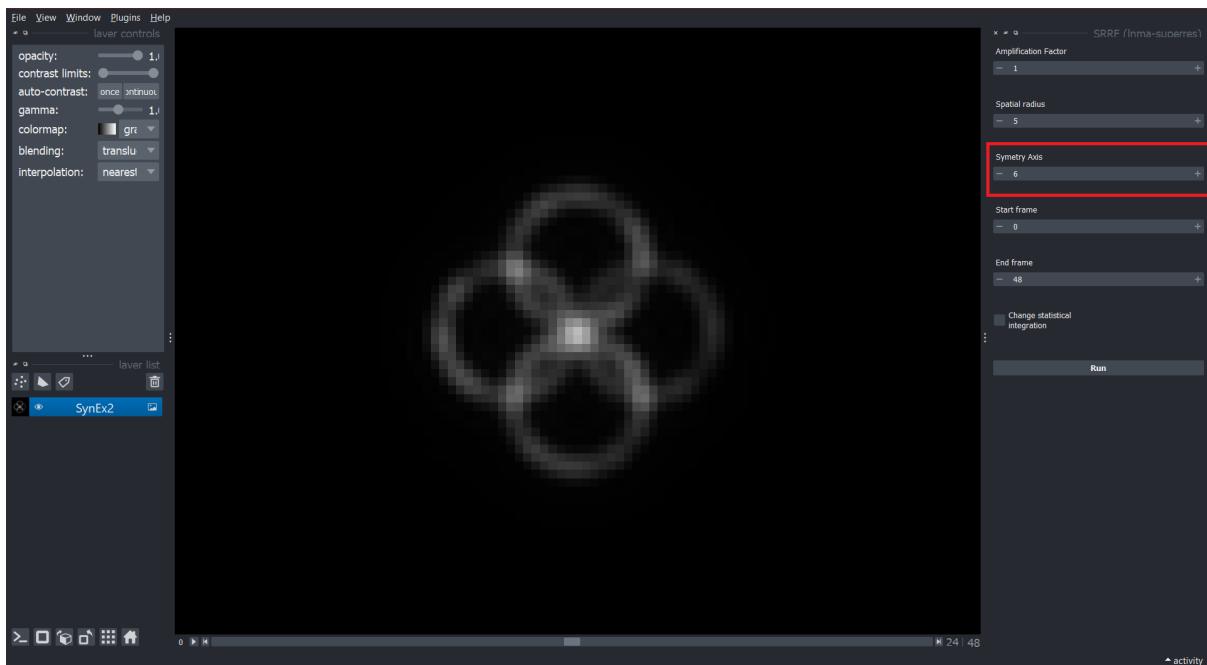
## 6.2 Spatial radius.

The ring radius parameter determines the size of the ring used to calculate the intensity gradient vectors of nearby subpixels. Optimal resolution is typically achieved with a smaller ring radius. However, it's important to consider the nature of the data. In busy or noisy datasets, using a small ring radius may reduce precision and introduce unwanted patterns. Generally, for sparse data, a smaller ring radius is suitable, while denser data may benefit from a larger ring radius. The default value in SRRF is a ring radius of 0.5. Changing the ring radius can significantly impact the final results, balancing resolution and noise patterns.



### 6.3 Symmetry Axis.

Radiality calculation involves analyzing a ring around each subpixel, and the size of this ring is determined by the ring radius. The axes parameter in the ring defines the number of points around the circumference of the ring that are utilized for the calculation. A higher value increases the fidelity of the data but also results in longer computational time. It is generally advised not to decrease this value below the default setting, which is 6.



## 6.4 Start and End Frame.

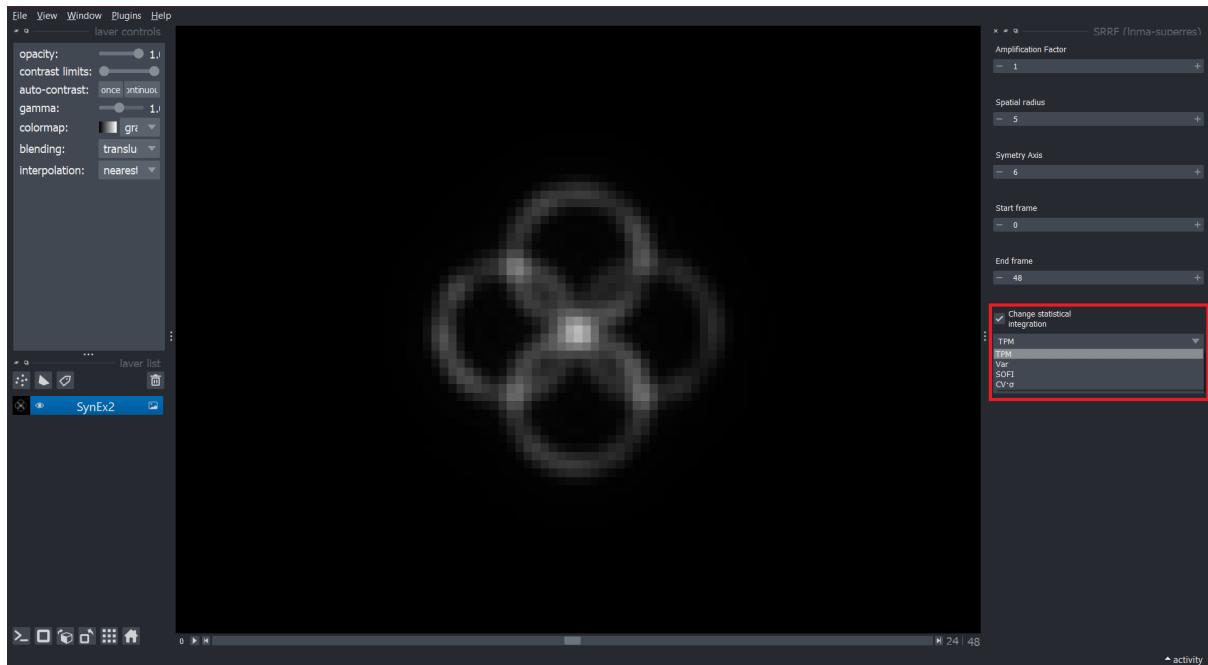
Within these options, users have the flexibility to select the specific range of images they wish to analyze using SRRF from an image stack.



And finally

## 6.5 Change statistical integration.

Lastly, we encounter the "Change Statistical Integration" option, which grants us the ability to adjust the desired temporal analysis. This feature offers several choices, including variance (Var), temporal product mean (TPM), coefficient of variation (CV), and auto-cumulant function (SOFI).



Once these parameters have been adjusted to your liking, simply click on the "Run" button to initiate the analysis with SRRF. It will then perform the subsequent image analysis based on the chosen options within napari. Sit back and let SRRF work its magic, unraveling finer details and enhancing the resolution of your images for further examination and exploration within the napari framework.



## 7. SOFI MANUAL

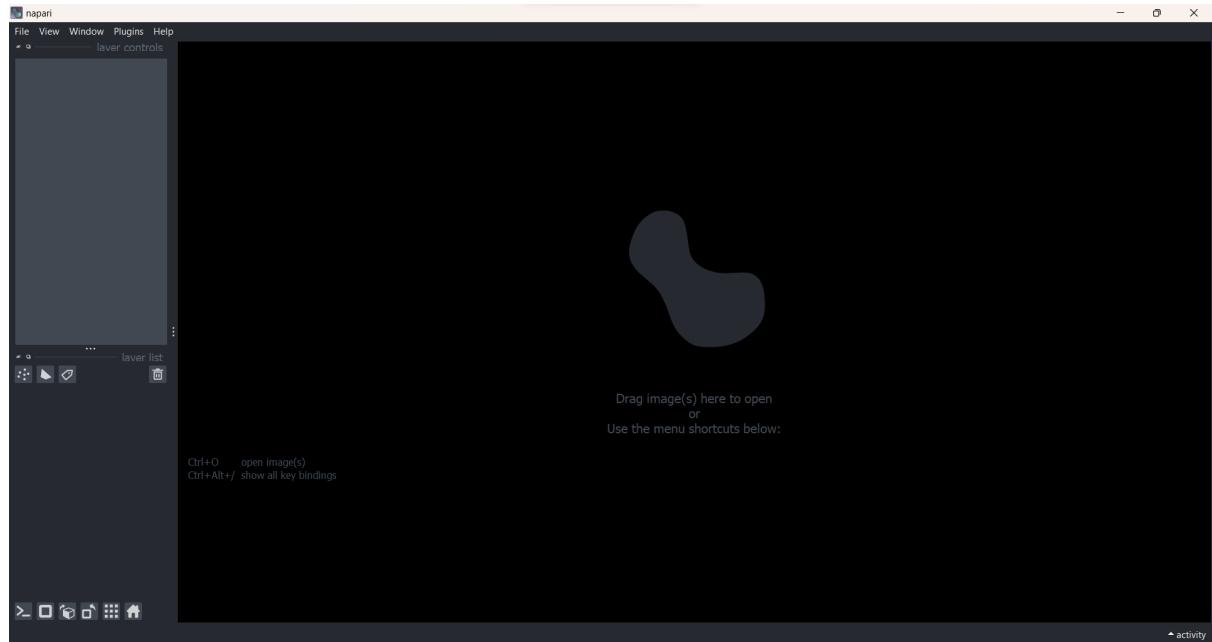
SOFI (super-resolution optical fluctuation imaging) is a powerful 3D super-resolution technique that surpasses the diffraction limit, producing high-quality images with minimal background noise. It achieves this by analyzing the temporal fluorescence fluctuations of individual emitters, eliminating the need for complex electronics or acquisition schemes. By simply capturing a movie of the sample, SOFI generates contrast-enhanced images in just a few seconds.

Although SOFI's deconvolution algorithms perform optimally on background-free images, one limitation is the scaling of brightness in the resulting images. As the order of SOFI increases, initial differences in brightness become more pronounced.

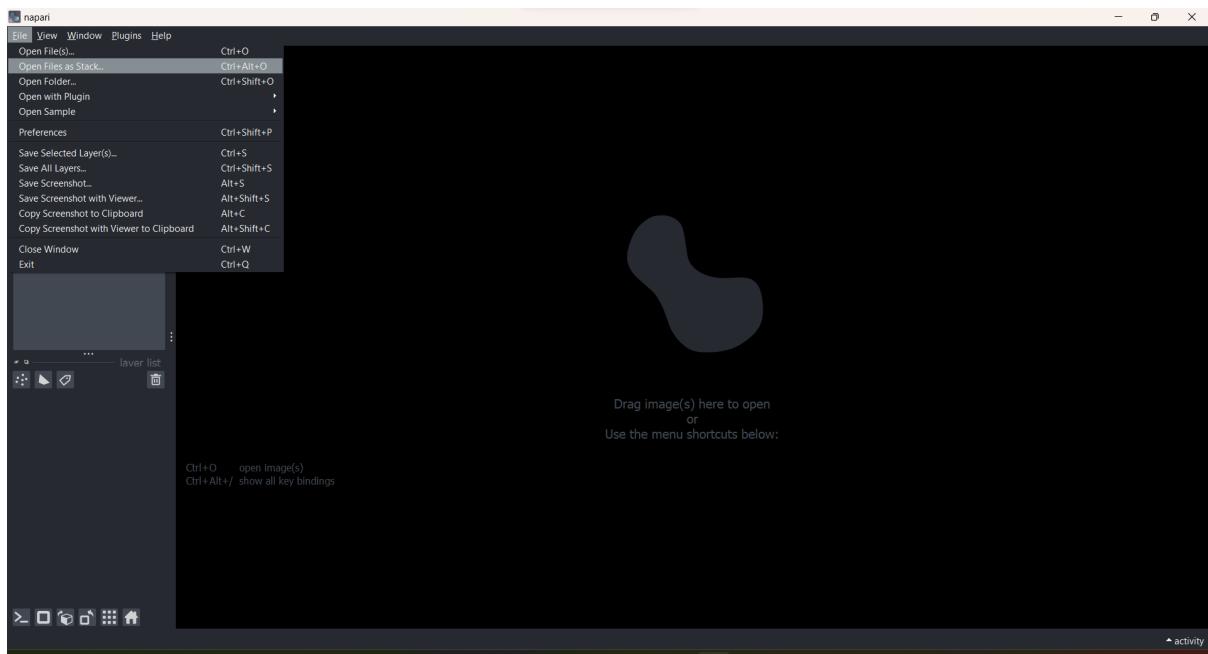
SOFI can be considered a single-molecule technique as it detects fluctuations from individual emitters. While it theoretically eliminates any type of noise due to its temporal correlation analysis, the signal-to-noise ratio remains a factor of concern, particularly due to limited acquisition times caused by factors like photobleaching. Its flexibility is evident as it can work with a range of microscope objectives, not limited to those with high numerical apertures. This versatility makes SOFI applicable in various imaging scenarios where its primary benefit lies in background reduction..

Now we will see how to apply the SOFI algorithm for processing stacks of images using Napari:

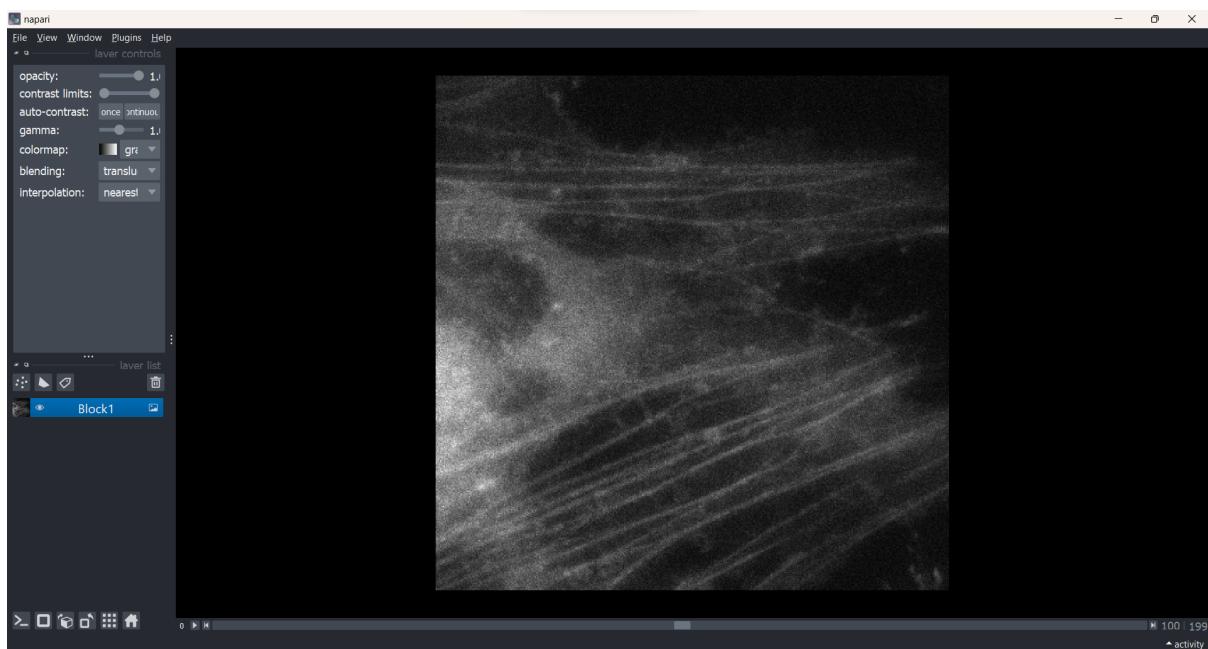
When starting Napari from the console, we will observe the interface with which we will interact throughout this manual



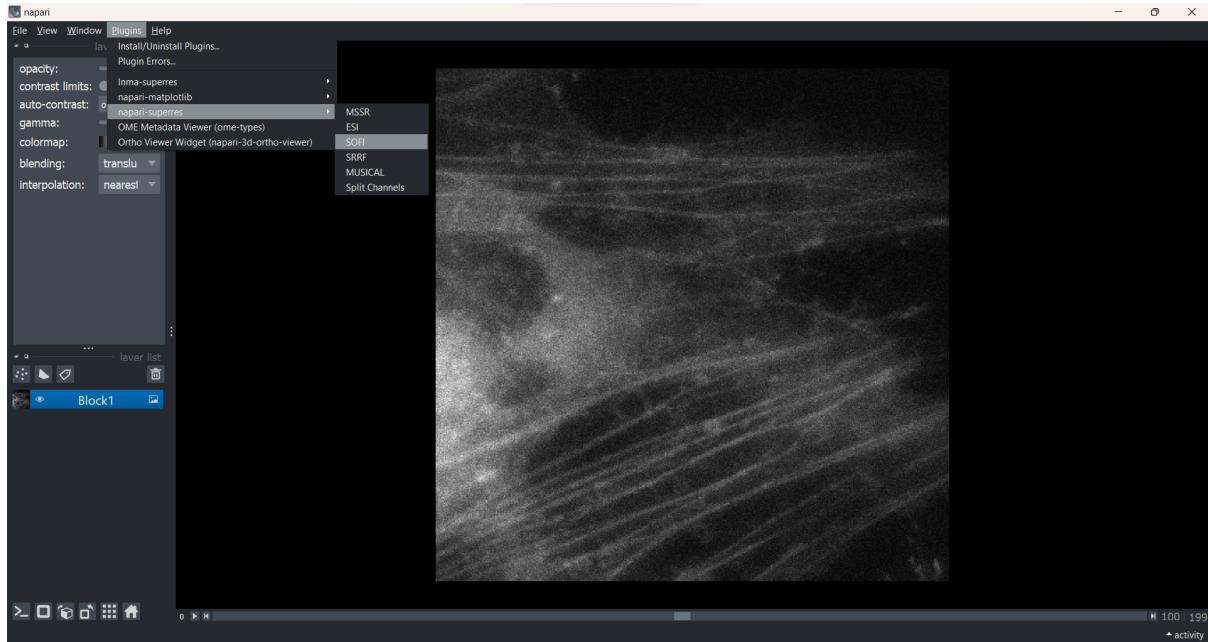
To open the image stack, we need to go to the "file" tab and select "Open Files as Stack" and choose our data file.



Now we will be able to visualize and navigate through the images that make up our data file. For example, here we are using a stack of 199 images for processing with SOFI.



Now we will process the image stack using the "napari-superres" plugin, where we will select the SOFI algorithm as shown in the following image:

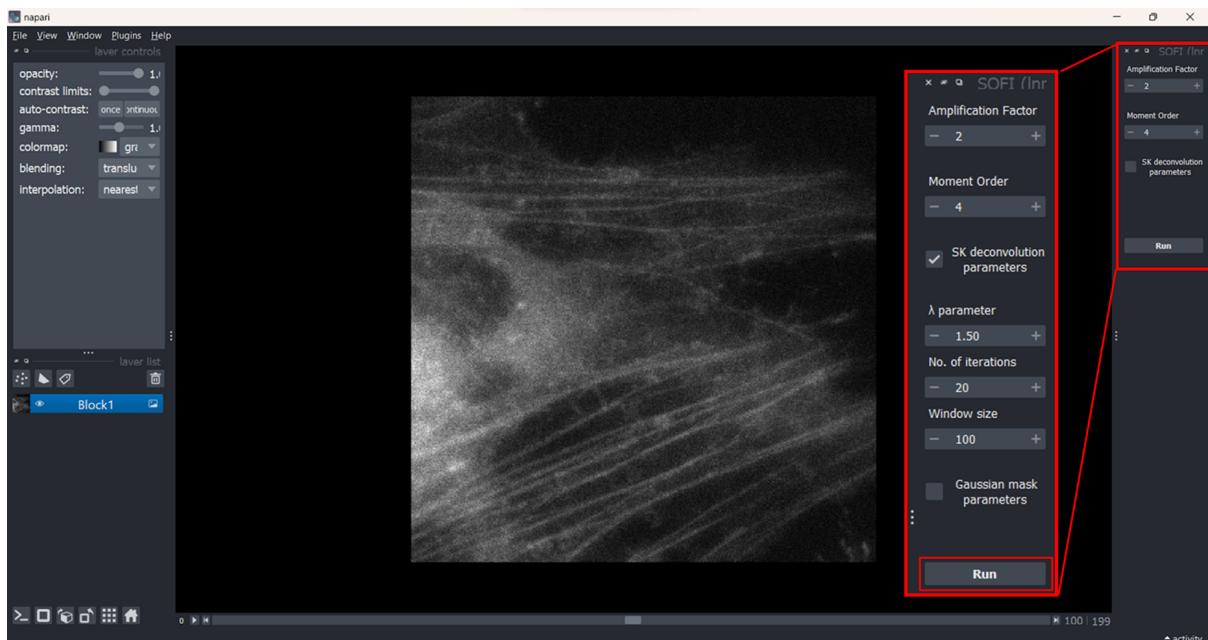


We proceed to select the parameters of the SOFI algorithm for processing the image stack. In this case, we select values of Amplification factor: 2 and Moment Order: 4. Later on, each parameter involved in the SOFI algorithm is described to understand the significance of each one.

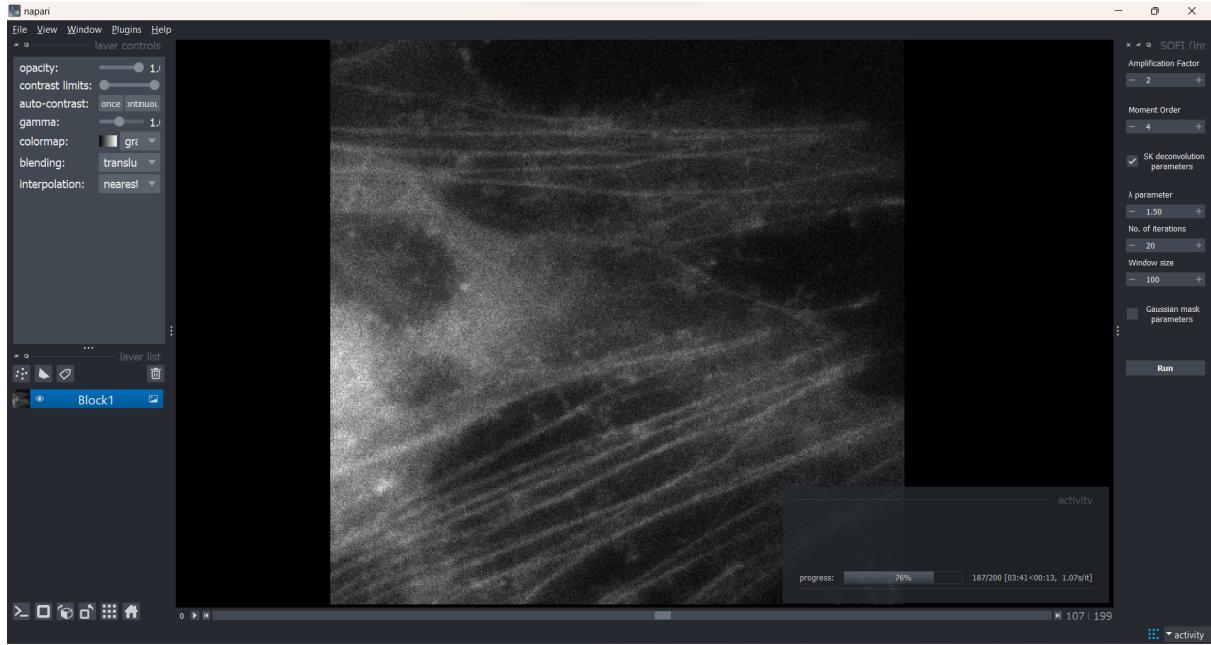
Then, select the SK deconvolution parameters and enter the following values:

- Lambda parameter: 1.5
- Number of Iterations: 20
- Window size: 100

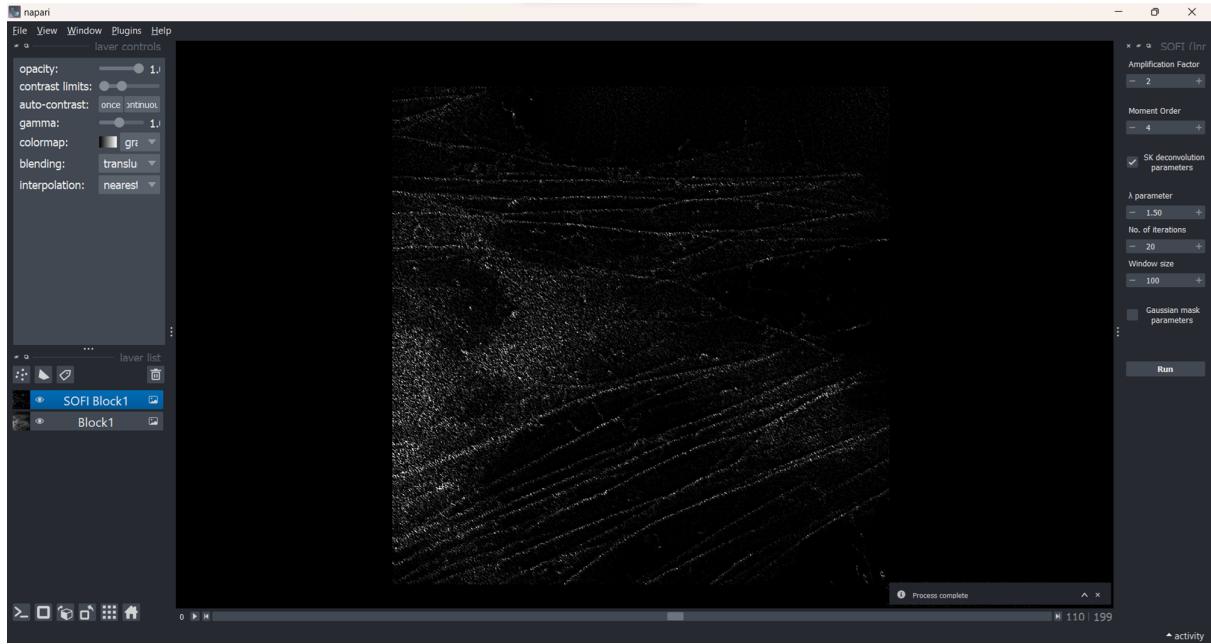
Next, click on "Run" to start the processing.



While the SOFI algorithm is running, we can monitor the progress in the "Activity" section, where we can see the percentage of completion.



Afterward, you will receive a notification indicating the completion of the process at this point. You can then select the layer to view the results.



However, you can adjust the values as needed. Here, we explain the principles behind each parameter:

### 7.1 Amplification factor.

The amplification factor parameter in image processing enhances the signal or features in an image. By adjusting this factor, desired details can be amplified while controlling noise and artifacts. Additionally, the amplification factor determines the number of subpixels used to

divide the original image pixels, leading to improved resolution. However, a higher number of subpixels increases computational time. Thus, selecting the appropriate amplification factor requires balancing resolution enhancement with computational efficiency.

## **7.2 Moment order.**

The moment order in the SOFI algorithm refers to the specific order of statistical moments used to describe properties of a data distribution. By incorporating higher-order moments, the algorithm enables the reconstruction of high-resolution images with finer details. These higher-order moments provide a more comprehensive analysis of the data, capturing additional information beyond lower-order moments. However, the choice of moment order involves a trade-off between computational complexity and desired image detail, requiring careful consideration based on imaging goals and constraints.

## **7.3 SK deconvolution parameters.**

In the SOFI algorithm, "SK deconvolution parameters" are specific parameters used to reverse blurring or degradation effects in the image. "SK" stands for "Shrinkage and Kernel," representing the core principles of this approach. Shrinkage reduces noise and artifacts by attenuating specific frequency components, while the Kernel relates to the point spread function (PSF) that characterizes blurring in the imaging system. These deconvolution parameters are essential for restoring image quality and enhancing the visibility of details.

Fine-tuning SK deconvolution parameters in the SOFI algorithm is crucial for achieving optimal results. These parameters can be adjusted to control noise reduction, preserve image details, and accommodate computational constraints. By carefully optimizing these parameters, the deconvolution process strikes a balance between noise reduction and feature preservation, ultimately producing high-quality output images.

## **7.4 $\lambda$ Parameter.**

The lambda parameter controls the level of shrinkage applied during the deconvolution process. Increasing the lambda value leads to more pronounced shrinkage and greater reduction of noise. However, there is a trade-off as higher lambda values can potentially result in the loss of fine details in the image. Finding the right balance for lambda is important to achieve effective noise reduction while preserving important image features.

## **7.5 No. of iterations.**

The number of iterations refers to the count of repetitions in the deconvolution process. With each iteration, the image is refined by iteratively applying the deconvolution operation. Increasing the number of iterations can enhance the image quality, but it also requires more computational time. The selection of the appropriate number of iterations involves a balance between achieving optimal image refinement and managing the computational resources available.

## **7.6 Window Size.**

The window size parameter determines the dimensions of the sliding window utilized during the deconvolution process. This window serves the purpose of analyzing the local properties of the image and plays a crucial role in estimating and adapting the deconvolution operation. By adjusting the window size, one can effectively capture and process the relevant

information in localized regions of the image, contributing to the accuracy and adaptability of the deconvolution process.