MSSR: Mean-Shift Super Resolution – Operation Manual

Release 1.0.0, 08/10/21

Correspondence: adan.guerrero@ibt.unam.mx

Disclaimer

We want to thank you for choosing to use this plugin.

The information in this document is designed to help you correctly use the MSSR plugin for ImageJ/FIJI. Read this entire document to understand how to define the parameters and the possible options when using this plugin.

The MSSR method was developed to work with fluorescence microscopy images, not work phase contrast images, nor light interference phenomena, nor special data such as Scanning Electron Microscope images (SEM), etc.

This plugin is designed to work with stacks of images, where each image represents the same area over time. If you have hyperstacks i.e. stacks of images with two or more channels, please first manipulate your data in this case split your data so that you have one stack of images per channel. If you decide to perform the analysis with that kind of hyperstacks the result probably will be a wrong interpretation of the data since in the data processing the hyperstack will be transformed into a conventional stack and the images will be rearranged.

TABLE OF CONTENTS

1. Downloading from GitHub

Prior to MSSR installation, make sure the latest version of FIJI is running on your computer (https://fiji.sc/).

Download the MSSR file from https://github.com/MSSRSupport/MSSR.

A) Click in MSSR_1.0.0.jar (Fig. 1A).
B) Click on the Download button (Fig. 1B).
C) The MSSR_1.0.0.jar file should be in your download directory (Fig. 1C).

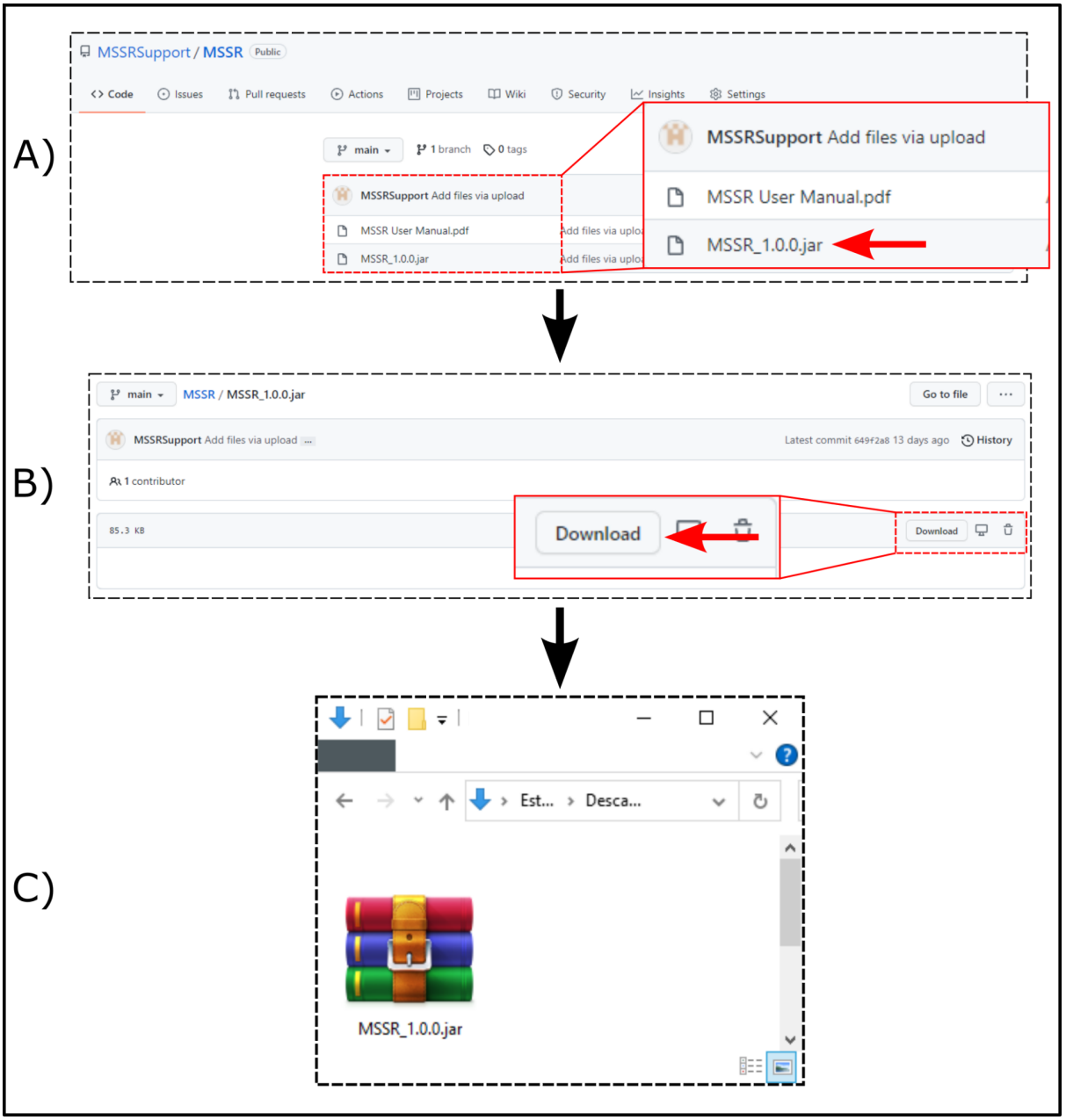

Figure 1. Scheme of download

2. Installation

1. Plugin installation can be done in two different ways:
    A. Through the installation option in FIJI (Plugins -> Install -> MSSR_1.0.0.jar) (Fig. 2A).
    B. By directly placing the MSSR_1.0.0.jar file in the corresponding plugins folder on FIJI (Fig. 2B).
2. Install the CLIJ, CLIJ2 and CLIJx packages (https://clij.github.io/clij2-docs/installationInFiji) (Fig. 2C).

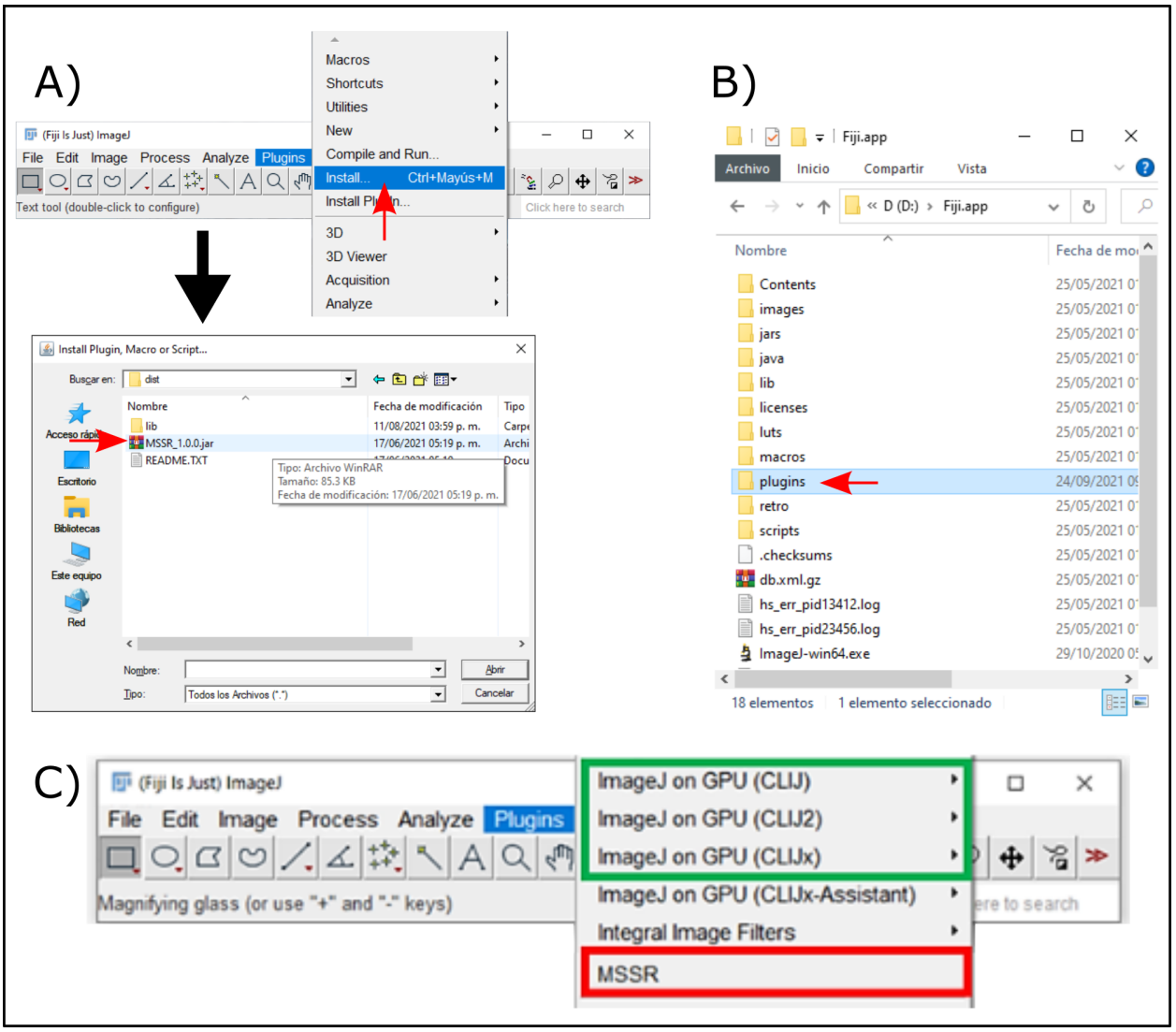

Figure 2. Installation steps

3. Description of the plugin

Two GUIs are available (Fig. 3A):

- *MSSR Analysis*
- *Temporal Analysis* (to perform over a preprocessed single-frame MSSR^n stack).

Three parameters are needed for the analysis (Fig. 3B.1) (to know the meaning of these parameters and how choose them to use, go to section 4):

- *AMP* – A digital zoom that you want to apply to the data
- *PSF* – The number of pixels that covers the distance for the Rayleigh Criterion
- *Order* – The number of MSSR iterations to increase the resolution of the image

MSSR Temporal analysis (Fig. 3B.3):

- Mean
- Variance (Var)
- Temporal Product Mean (TPM)
- Coefficient Variation
- Auto-cumulant Function of order 2-4 (SOFI 2-4)

Additional features:

- *Computation of the optical system's PSF* – automatically provides an estimation of the image PSF (in pixels) based on the optical parameters (Fig. 3C)
- *Minimize Meshing* – Enable the mesh minimization algorithm to reduce the 'mesh' pattern that possibly appears during the analysis (Fig. 3B.2)
- *GPU Computing* – Enables GPU usage for enhanced computing times (Fig. 3B.2)
- *Selecting Image*– Select an image or stack which is already open in FIJI (Fig. 3B.4)
- *Batch Analysis* – Allows to automatically analyze all the images in a given folder in your system (Fig. 3B.4)

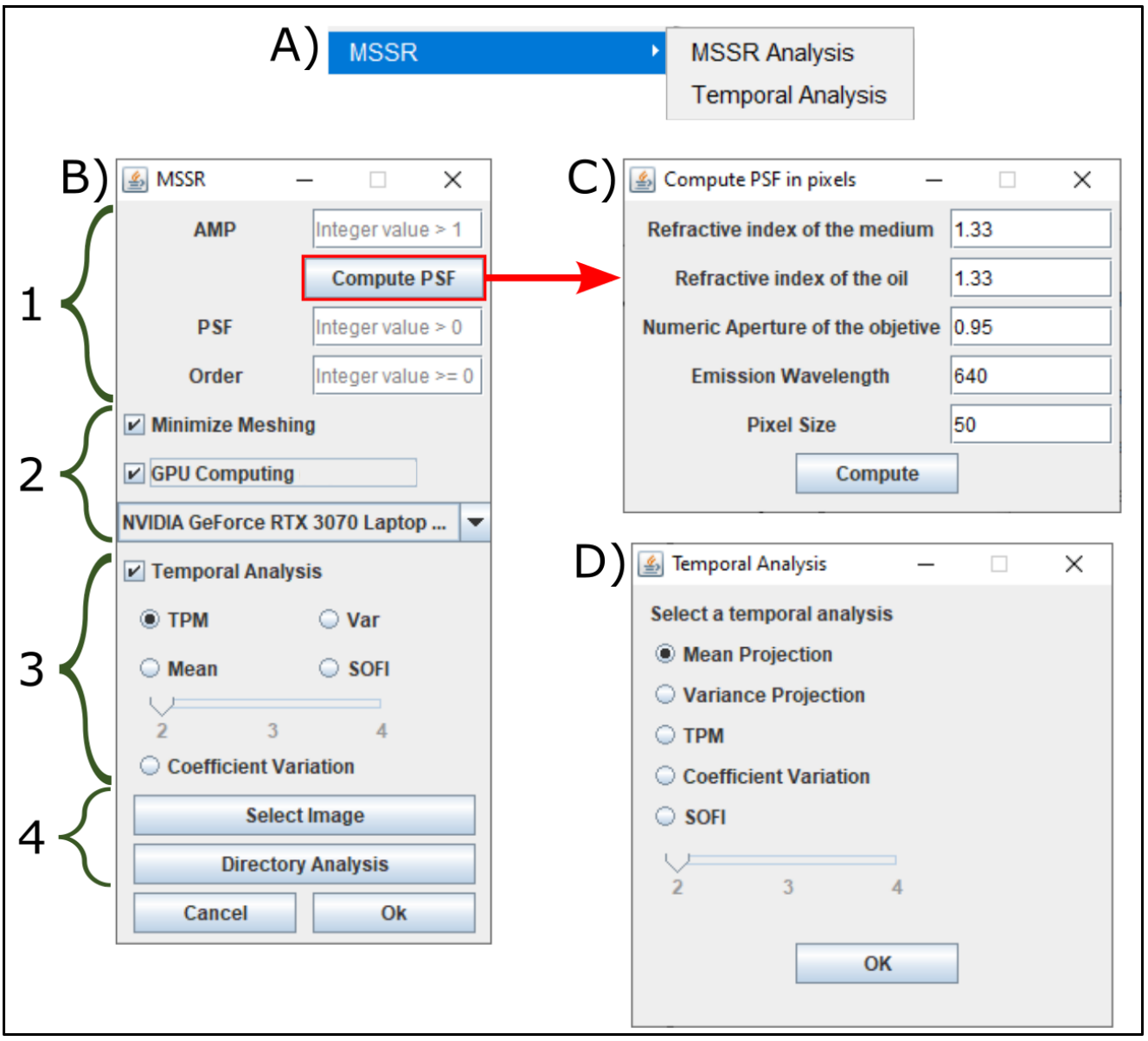

Figure 3. Available GUIs

4. Meaning of the parameters of MSSR and how to choose them

4.1. Amplification value (AMP)

The magnification value defines the digital zoom that will be applied by interpolating the image, to generate sub-pixel information. This parameter takes integer numbers equal to or greater than 1.

If you have an image with N x M dimensions with a pixel size of 20 nm (1px equal to 20nm) and an amplification value of 5 is used, then an image with (5*N) x (5*M) dimensions with a pixel size of 4nm is obtained (5px equal to 20nm) (Fig. 4).

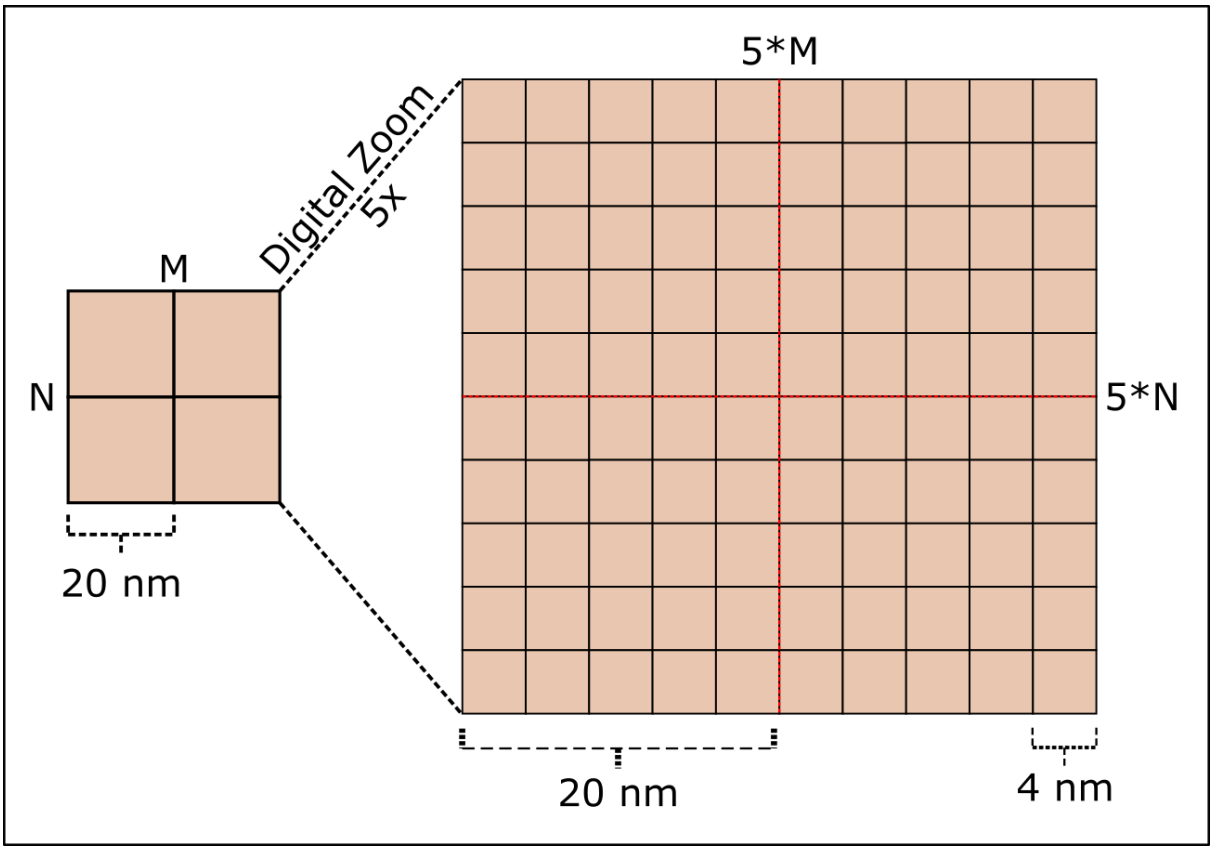

Figure 4. Scheme of the effect of the amplification value (digital zoom)

To choose which value to use, you can simply solve the next equation based on the resolution (in pixel size) you want to achieve.

$$\frac{Current\ Pixel\ Size}{Desired\ Pixel\ Size} = Amplification\ value \qquad \text{Eq. 1}$$

4.2. Point Spread Function (PSF) (For more details, see the supplementary note 1)

The PSF is a function that defines the distribution pattern of the photons when passing through an optical system. The result of convolving the PSF with a single emitter is an Airy pattern in which approximately 86% of the emitted light is harbored within the central disk. Concentric rings are arranged around the central disk whose intensity decreases as a function of the distance from the center.

Here, the value of PSF is defined as the number of pixels that covers the distance from the center point of the distribution to the first minimum of the distribution (first dark ring) (Rayleigh Criterion) (Fig. 5). This parameter takes real numbers greater than 0.

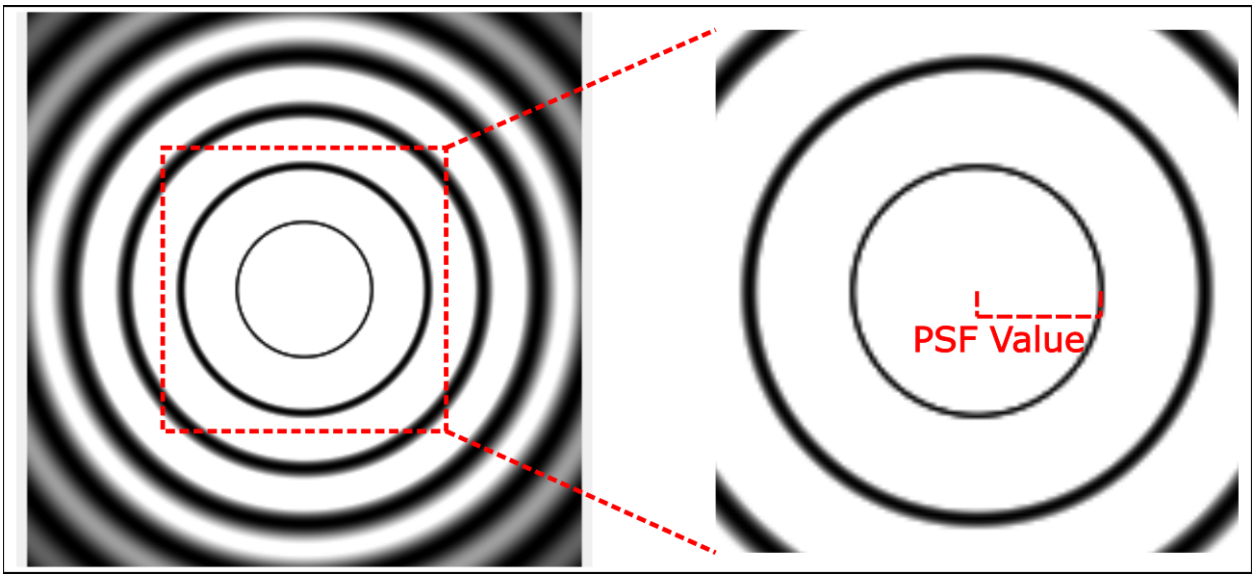

Figure 5. Scheme of the meaning of the PSF value for the plugin

To choose which value to use, you can solve the next equations using the experimental parameters, such as:

- **Refractive Index of the Medium** - The refractive index of the medium where the sample is located.
- **Refractive Index of the Oil** – The refractive index of the medium between the lens and the coverslip.
- **NA Objective**– The numerical aperture of the lens used.
- **Wavelength of Emission** – The wavelength of emission of the fluorophores (in nanometers), if the fluorophores emit under a spectrum, then use the maximum value of that range.
- **Pixel Size of the image** – The distance that one pixel represents in the image (in nanometers).

$$NA = \frac{Refractive\ Index\ of\ the\ Medium}{Refractive\ Index\ of\ the\ Oil} * NA_{Obj} \quad \text{Eq. 2}$$

$$Rayleigh\ Criterion = 0.61 * \frac{Wavelength\ of\ Emission}{NA} \quad \text{Eq. 3}$$

$$\#Pixels_{PSF} = \frac{Rayleigh\ Criterion}{Current\ Pixel\ Size} \quad \text{Eq. 4}$$

$\#Pixels_{PSF}$ represents the number of pixels that the PSF of the optical system covers in the image and is the value to use in the PSF parameter of the plugin. Another option is to use the "Compute PSF" button where you need to feed these values and the plugin will calculate the corresponding PSF value.

Suppose you have a sample with FM4-64 fluorophores (maximum wavelength of 640 nm), immersed in water (refractive index of 1.33) and we use a water immersion objective with a NA of 0.95, and a pixel size of 50 nm, then you will obtain a value of 8.22 for the PSF parameter.

4.3. Order (For more details, see the supplementary note 5.1)

The MSSR analysis consists of two main processes:

- MSSR of order 0 ($MSSR^0$)
- MSSR of higher orders ($MSSR^n$, n > 0)

The "Higher Order" encompasses a sequence of iterative operations which increase the resolution of the image. These iterative operations are carried out with the two previous results of the analysis ($MSSR^{n-2}$ and $MSSR^{n-1}$), in the case of $n = 1$ the $MSSR^{n-2}$ is the original image since there is no order before $n = 0$ (see Fig. 2a main article). The order is commonly set to a maximum of 2 or 3, given that part of the emitter's information is degraded with each iteration (supplementary note. 5.1.2).

4.4. Temporal Analysis (For more details, see the supplementary note 5.2)

The MSSR process is applied to a single image (before the temporal analysis), that means that if you analyze a stack of images, before the temporal analysis is performed, what you have is a sf-MSSR^N stack, then the temporal analysis is perform applying a pixel-wise function (Pixel-wise Temporal Function or PTF) to de data in the t-axis, obtaining a single image t-MSSR^N (Fig. 6).

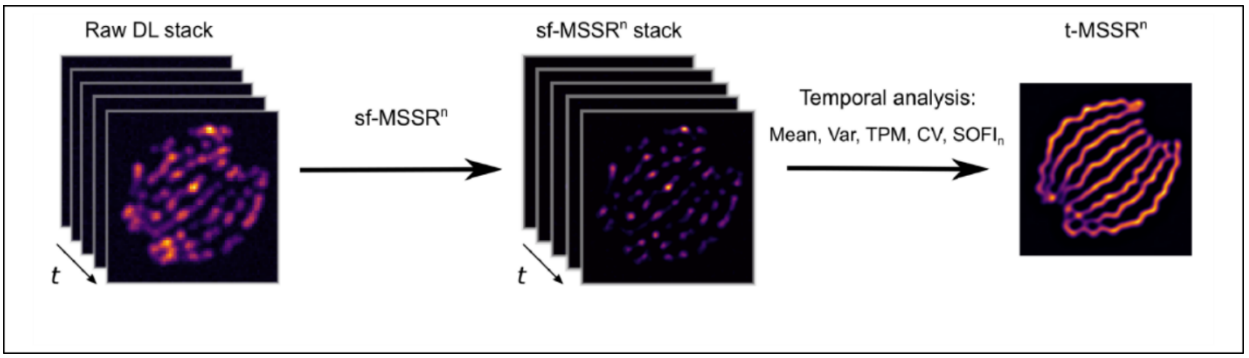

Figure 6. Scheme of the MSSR Analysis

To choose which temporal analysis to use you can use the blinking nature of your fluorophores (Fig. 7) (Table 1).

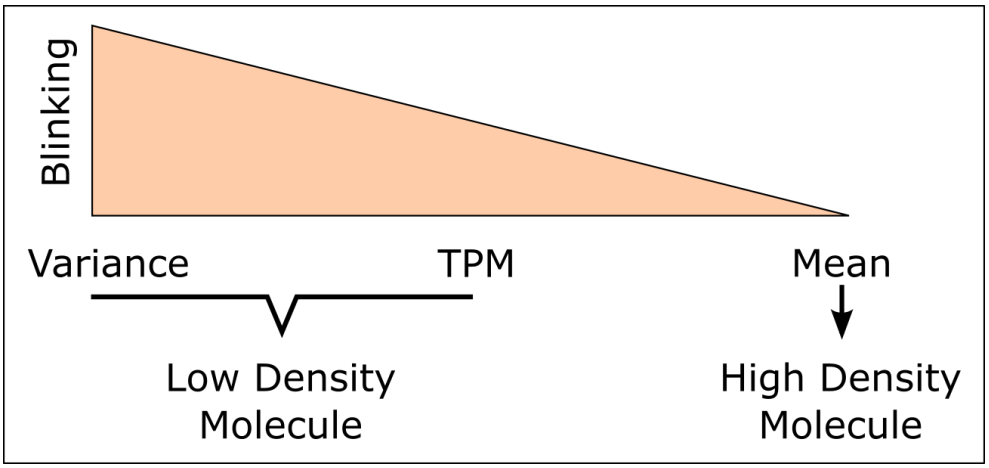

Figure 7. Scheme of the temporal analysis in function of the blinking

PTF Notation	Name	Recommended Usage
Mean	Average	Low blinking of fluorophores
TPM	Temporal Product Mean	Intermediate blinking of fluorophores
Var	Variance	High blinking of fluorophores
CV	Coefficient of Variation	
$SOFI_2$	Auto-cumulant order 2	
$SOFI_3$	Auto-cumulant order 3	
$SOFI_4$	Auto-cumulant order 4	

Table 1. MSSR temporal functions and their recommended usage.

4.5. Minimize Meshing (For more details, see the supplementary note 6)

It was found that the interpolation process generates an artifact with a mesh pattern and that the appearance of this artifact is related to the presence of noise in the image.

Due to noise being intrinsically and inevitably introduced along the imaging process in any optical system, the 'meshing' effect will always take place when real experimental data is analyzed (supplementary note 6.4).

Therefore, the option "Minimize Meshing" is always selected when the plugin is opened, and we encourage the analysis to be carried out with this option enabled.

4.6. GPU Computing (For more details, see the supplementary note 7)

This option allows the plugin using the CLIJ package to scan the available GPUs in your computer so that you can choose one and perform the analysis with that GPU, allowing to speed up the data processing time to a great extent (depending on the GPU and the parameters used).

4.7. Batch (Directory) Analysis

This option allows selecting a directory that contains multiple data with a single image, to carry out the analysis with the parameters previously defined in all the data, generating a folder that contains the results of the MSSR analysis for each file.

In this option, if the directory contains a file with more than one image, this file will be ignored when performing the analysis. This feature is designed to analyze many single image data with the same set of parameters (AMP, PSF, Order).

5. Usage

Example of MSSR Temporal Analysis:

When selecting a temporal analysis, two images will be generated: (**i**) a single-frame MSSR^n stack of the original data and (**ii**) the temporally analyzed, super-resolved frame. If only a single-frame MSSR analysis was previously performed over a diffraction-limited stack, one can choose to additionally perform a temporal analysis without re-running the whole process. For the latter, choose the ***Temporal Analysis*** GUI from the plugin menu. Parameters used for this example: AMP = 5, PSF = 3, Order = 1, Meshing minimization = Yes, GPU computing = Yes.

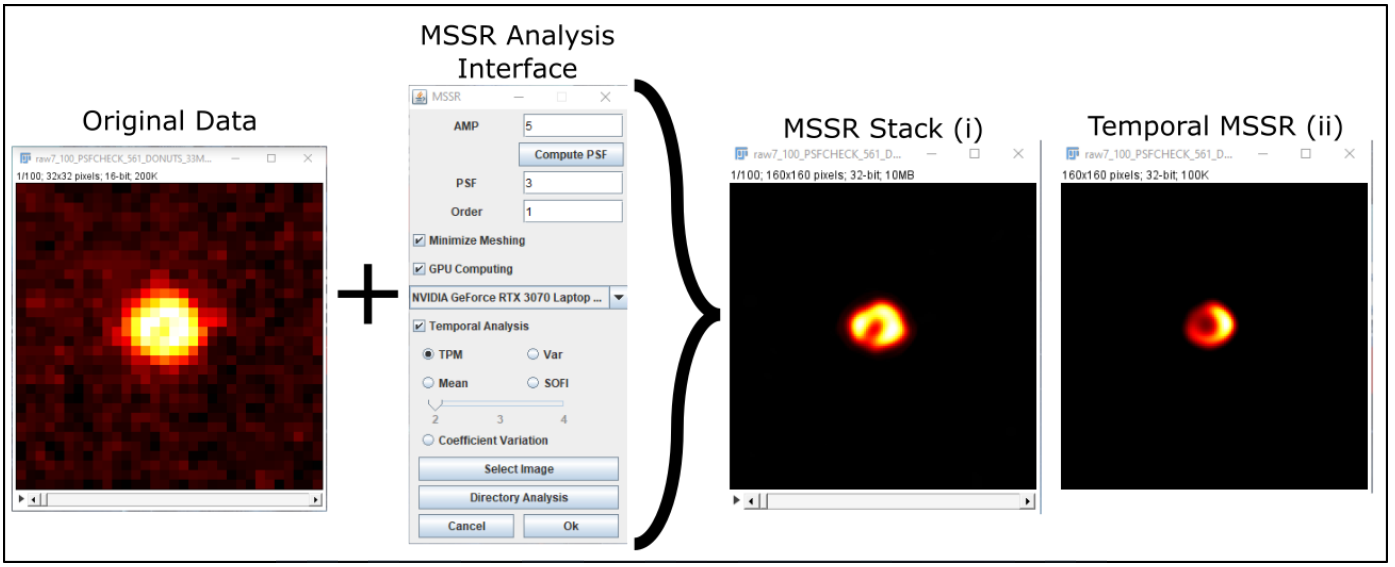

Figure 8. Scheme of MSSR Analysis Interface usage

Example of Batch (Directory) Analysis:

Selecting the *Batch Analysis* button will open a new window, where the path to analyze is specified; the selected path will be displayed on the GUI. Once the analysis has finished, a folder called "MSSR" will be created within the analyzed directory and will contain all the resulting super-resolved MSSR images.

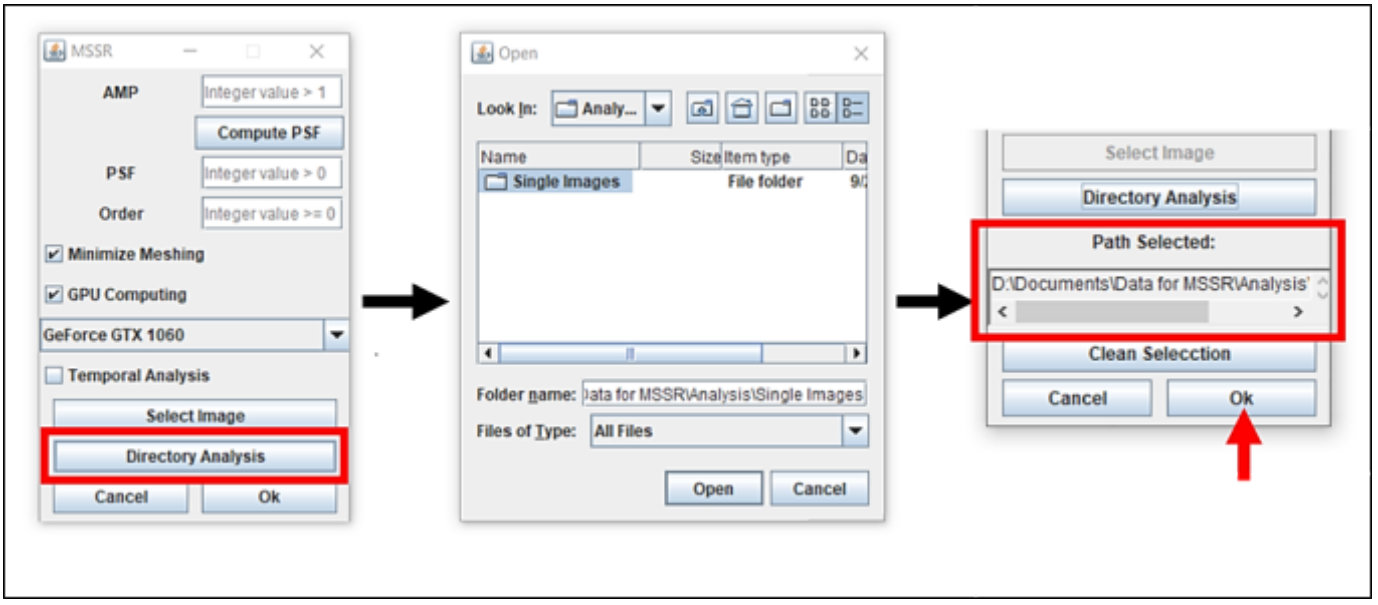

Figure 9. Scheme of Batch Analysis usage

6. Applications

(For more details, see the supplementary note 10)

There are multiple scenarios in which MSSR analysis can be used; these scenarios include but are not limited to:

- Immunofluorescence imaging of fixed cells by epifluorescence microscopy.
- Live-cell imaging using permeable dyes with total internal reflection fluorescence microscopy.
- Single particle tracking.
- Single-molecule localization microscopy.
- Colocalization microscopy.
- Tissue live-cell imaging - laser scanning confocal microscopy.